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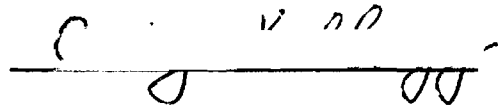

THE CHEMISTRY OF VIOMYCIN

A THESIS

Presented to

The Faculty of the Graduate Division

by

Craig Kent Kellogg

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

in the School of Chemistry

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April, 1963

THE CHEMISTRY OF VIOMYCIN

Approved:

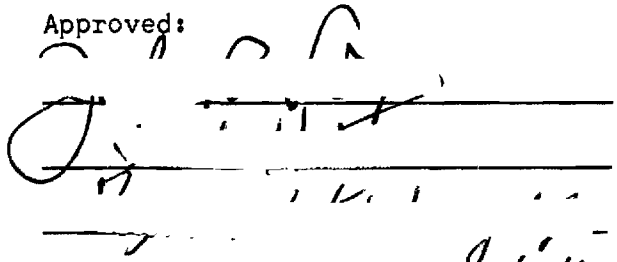

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TABLE OF CONTENTS

LIST OF TABLES

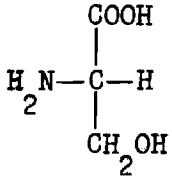
LIST OF FIGURES

SUMMARY

Viomycin is a potent, water soluble antibiotic that was isolated simultaneously in the laboratories of Chas. Pfizer & Company and Parke, Davis & Company. Subsequent work demonstrated that the two isolates were identical and that viomycin is toxic.

The purpose of the present research was to determine as many of the structural features of viomycin as possible. The ultimate goal of investigations of viomycin will be to determine its structure in complete detail.

Previous investigation of viomycin has shown that it is a polypeptide. Acid hydrolysis of viomycin gives L-serine (I), L-β-lysine (II), L-2,3-diaminopropionic acid (III), urea, carbon dioxide, ammonia, and a mixture of strongly basic compounds. The structure of viomycidine (IV), the principal compound present in this mixture, recently has been elucidated.



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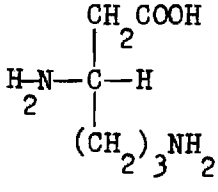

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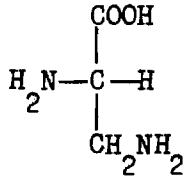

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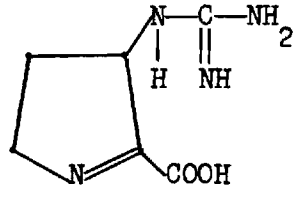

IV

Of the information required for deducing the structure of a polypeptide, the most basic is the order of peptide linkages. Determination of this order of peptide linkages requires the following information: the molecular weight of the peptide or protein; the

identity and amount of each amino acid released on complete hydrolysis; the determination of C-terminal and N-terminal amino acid residues; and partial hydrolysis to peptides of lower molecular weight that can be identified.

In addition to incorporating the order of peptide linkages, any structure proposed for viomycin will have to explain its ultraviolet absorption spectrum, its nuclear magnetic resonance spectrum, and the presence of urea, carbon dioxide, and ammonia in its hydrolysate.

Some of the information required for the determination of the structure of viomycin had been obtained prior to the present work. The molecular weight was determined by the diaphragm-cell diffusion technique. A molecular formula, based on molecular weight and analysis, had been proposed. Titration data and the molecular formula showed that viomycin has three basic centers, whose pK_a values are 8.2, 10.3, and ca. 12, and no acidic centers. Thus viomycin has no carboxyl group. However, the facts that viomycin has weakly basic centers (pK_a 8.2 and 10.3) and that it has two primary amino groups (van Slyke) suggested that it might have one or more N-terminal amino acid residues. Preparation and hydrolysis of the N-2,4-dinitrophenyl derivative of viomycin had shown that at least one of the amino groups of the β-lysyl residue of viomycin was free. One of the goals of the present research was to prepare and further characterize the N-2,4-dinitrophenyl derivative of viomycin. Various conditions for hydrolysis of the N-2,4-dinitrophenyl derivative were explored and the hydrolysates were investigated. Paper chromatography indicated that hydrolysis with concentrated hydrochloric acid gave bis-2,4-dinitrophenyl-β-lysine as the only dinitrophenyl

compound. Bis-2,4-dinitrophenyl-β-lysine was isolated, purified, and its identity established by its melting point and the melting point of mixtures of it and a synthetic sample. This showed that the two primary amino groups of viomycin are those of the β-lysyl residue. Thus β-lysine is the N-terminal amino acid of viomycin.

Previous work had shown that viomycin has a guanidine group, an olefinic double bond or some other easily oxidizable group, and possibly an aldehydic carbonyl group. The presence of the guanidine group had been indicated by titration data (pK_a ca. 12) and by the fact that viomycin gives a positive Sakaguchi test. Oxidation of viomycin by neutral potassium permanganate had shown that viomycin has an olefinic double bond or some other oxidizable group. The presence of the olefinic double bond was confirmed by ozonolysis. Negative results with thiosemicarbazide and Fehling's reagents showed that viomycin has no aldehydic or ketonic carbonyl group. The possibility that viomycin has the Δ^1-pyrroline structure of viomycidine probably is eliminated by titration data (pK_a 5.50) and negative results with o-aminobenzaldehyde reagent.

Partial hydrolysis studies on viomycin have had only limited success. Of the conditions explored previously, treatment of viomycin with hot dilute acid was the most successful. In this way viomycin was hydrolyzed to urea and a new compound, desureaviomycin. Total hydrolysis of desureaviomycin gave serine, β-lysine, 2,3-diaminopropionic acid, viomycidine, carbon dioxide, and ammonia, but no urea. No promising results were obtained using new conditions for partial hydrolysis of viomycin. Therefore, desureaviomycin was characterized further and

subjected to partial hydrolysis conditions. It was found that desureaviomycin, unlike viomycin, had a free carboxyl group. This carboxyl group was shown by hydrazinolysis to be the carboxyl group of a seryl residue. Desureaviomycin was found to have two amino groups (van Slyke). The N-2,4-dinitrophenyl derivative of desureaviomycin was prepared and hydrolyzed to give bis-2,4-dinitrophenyl-β-lysine as the only dinitrophenyl compound. Thus the primary groups of desureaviomycin are those of its β-lysyl residue. These results show that the carboxyl group of a seryl residue, urea, and no new free amino groups are released in the conversion of viomycin to desureaviomycin.

Treatment of desureaviomycin with the enzyme carboxypeptidase gave serine and a new material, called viomycinic acid. Total acid hydrolysis of viomycinic acid gave serine, β-lysine, 2,3-diaminopropionic acid, viomycidine, and ammonia. Hydrazinolysis of viomycinic acid showed that the carboxyl group of a seryl residue was free. Preparation and hydrolysis of the N-2,4-dinitrophenyl derivative of viomycinic acid showed that both amino groups of its β-lysyl residue were free.

Viomycin has an absorption peak in the ultraviolet region. The position and intensity of the peak depend on the pH of the solution. In dilute acid the peak is at 268 mμ(ε, 23,300); in neutral solution the peak is at 268.5 mμ(ε, 22,900); and in dilute alkali the peak is at 282 mμ(ε, 14,600). Study of spectra of viomycin in solutions of pH 10, 11, 12, 13 and 14 had revealed that the guanidine group is involved in the ultraviolet chromophore. The chromophore is destroyed in acid hydrolysis. The fact that the chromophore is destroyed when viomycin

is treated with either neutral permanganate or ozone implies that the olefinic double bond also is involved in the chromophore.

Nuclear magnetic resonance spectra of viomycin, desureaviomycin, and viomycinic acid have an absorption at low values of τ(1.93-2.03). This absorption corresponds to one non-exchangeable proton in each case. None of the products of total hydrolysis of viomycin display absorption in this region.

The data obtained are discussed in terms of a tentative partial structure for viomycin.

INTRODUCTION AND HISTORICAL

Isolation and Clinical Studies

Viomycin was isolated simultaneously in the antibiotic soil screening programs of Chas. Pfizer & Company and Parke, Davis & Company from similar cultures of Streptomyces with red-violet mycelia, called Streptomyces puniceus and Streptomyces floridae, respectively (1,2). Preliminary data on the isolates indicated that the antibiotic samples were similar. Subsequent exchange of samples confirmed the identity of the two isolates.

Early biological studies indicated that viomycin would be useful as an antibiotic if human toxicity were low, since it was effective in the protection of mice against Mycobacterium tuberculosis H37Rv (1). However, administration of viomycin to humans with advanced tuberculosis over periods of two to ten weeks caused kidney damage, electrolyte imbalance, vestibular dysfunction, and hypersensitivity (3). Since it has these toxic effects, and since better antibiotics have been discovered, viomycin finds only limited clinical use.

Properties of the Intact Molecule

Viomycin is a strong organic base that forms essentially neutral salts with hydrochloric and sulfuric acids (1). Titration data indicate pK_a* values 8.2, 10.3, and 12 (1,4). Salts of viomycin are soluble in water, but not in organic solvents.

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*$pK_a = -\log (H^+)(A^n)/(HA^{n+1})$, where either A^n or HA^{n+1} can be the compound in question or the compound in a different state of protonation.

Viomycin exhibits one strong absorption peak in the ultraviolet region. The position and intensity of the peak depend on the pH of the solution. In 0.1 N hydrochloric acid the peak occurs at 268 mμ, (ϵ, 23,300); in neutral solution the peak is at 268.5 mμ, (ϵ, 22,900) and in 0.1 N sodium hydroxide the peak is at 282.5 mμ, (ϵ, 14,600). Comparison of ultraviolet spectra of viomycin in solutions of pH 10, 11, 12, 13, and 14 respectively, reveals isosbestic points at 235 and 281 mμ. The fact that there are two isosbestic points means that one dissociating group is involved in the chromophore (4,5). Evaluation of the pK_a of this dissociating group from these spectra gave a value of 12.4 (4). Thus it appears that the guanidine group is involved in the chromophore. The ultraviolet spectrum of viomycin was not changed when viomycin was exposed to catalytic hydrogenation conditions. Apparently no readily reducible group is involved in the ultraviolet chromophore (4).

Viomycin is optically active. The specific rotation of air-dried viomycin sulfate is -32° (one per cent solution in water) (1). When viomycin sulfate was dried in vacuo at 50°, the weight loss was 9.45 per cent and the specific rotation was -40.6°, (one per cent solution in pH 6.0 buffer) (2).

The empirical formula $C_{18}H_{31-33}N_8O_8$ has been indicated for the free base of viomycin by analysis of its sulfate, chloride, picrate, and reineckate salts (6). The molecular weight of the free base, as determined by the diaphragm-cell diffusion technique, was 625 (4). Therefore, the molecular formula of the free base is probably $C_{25}H_{44}N_{12}O_{11}$ (MW, 688). The formula $C_{25}H_{44}N_{12}O_{11} \cdot 3/2\ H_2SO_4$ (MW, 836)

has been reported by Ciba for Vinactin A, which is identical to viomycin sulfate (4).

Early work showed that viomycin is a polypeptide. Thus it gives a positive biuret test and resists mild acid hydrolysis (6). Incubation of viomycin sulfate in 1 N hydrochloric acid for eleven days at 37° reduced the microbiological activity to 25 per cent of the original value, but did not cause the release of appreciable amounts of amino acids. However, when a solution of viomycin sulfate in 6 N hydrochloric acid or in 6 N sulfuric acid is boiled for six hours, free amino acids are released (Table 1).

Color tests and reaction with periodic acid indicate that viomycin does not contain a carbohydrate moiety. Viomycin gives positive Sakaguchi, ninhydrin, and Fehling's tests, but negative Benedict's and maltol tests (1,2). These tests indicate the presence of a guanidine group and possibly an aliphatic aldehyde group, but no streptose or other maltol-forming entity. The presence of the guanidine group also is indicated by the strongly basic center (pK_a 12) of viomycin. Rapid oxidation of viomycin by neutral potassium permanganate indicates the presence of an ethylenic double bond, an aldehyde group, or other easily oxidizable function (4). Viomycin has a van Slyke amino nitrogen value of 1.57. This value indicates that two primary amino groups are present (6).

Degradation of Viomycin

Viomycin is easily degraded by alkali, but is quite resistant to mild acid (2). However, treatment with boiling 6 N hydrochloric or 6 N sulfuric acid releases amino acids as shown by the data in Table 1 (6).

The ultraviolet chromophore of viomycin is destroyed during this acid hydrolysis. At least seven compounds are released during the hydrolysis of viomycin. The relative amounts of these compounds present after hydrolysis with boiling 6 N hydrochloric acid for six and sixteen hours are shown in Table 2.

Table 1. Evidence for the Release of Amino Acids from Viomycin[a]

	Ninhydrin CO_2	Van Slyke Amino N	Reduction of Periodic Acid
Viomycin	0.0	1.57	0.7 (three hours at pH 4.4)
Hydrolysate	3.7	5.8	3.0 (ten minutes at pH 4.7)

a. All values expressed as moles per mole of viomycin.

Table 2. Compounds Released by Hydrolysis of Viomycin[a]

Compound	16 Hours (6)	6 Hours (4)
Carbon Dioxide	0.88	0.50
Ammonia	.92[b]	.70[b]
Urea	.40[c]	.74[d]
L-2,3-Diaminopropionic Acid	.14[e]	.42
L-β-Lysine	.44	.90
L-Serine	1.30	---
Viomycidine	.16	.21

a. All values expressed as moles per mole of viomycin.

b. Ammonia was determined as volatile base by aeration into boric acid.

c. Urea was determined by conversion to ammonia by urease followed by determination of the resulting ammonia.

d. Urea was determined by the biacetylmonoxime method of Ormsby (7).

e. The amounts of the amino acids were determined by isolation.

Two-dimensional paper chromatography of the hydrolysate revealed that a second guanidine compound was present. Urea and the amino acids were isolated from the acid hydrolysate by ion exchange chromatography (6).

Urea was isolated as the dixanthydryl derivative (6). L-Serine was identified by optical rotation, analysis, and by preparation of the N-2,4-dinitrophenyl derivative (6).

L-2,3-Diaminopropionic acid was identified as the monohydrochloride salt by analysis for carbon, hydrogen, nitrogen, ionic chloride, van Slyke amino nitrogen, and ninhydrin carbon dioxide. It was assigned the L-configuration from its optical rotation and the optical rotation of the N,N'-dibenzoyl derivative (4).

An isomer of lysine was isolated from the acid hydrolysate of viomycin (6). This isomer was shown to be identical to L-3,6-diaminohexanoic acid, which also is obtained from streptothricin hydrolysates (8) and from streptolin hydrolysates (9). This compound was given the trivial name L-β-lysine.

The hydrolysate of viomycin was found to contain a mixture of strongly basic compounds that gave positive Sakaguchi tests (4,6). Viomycidine, the principal compound of this mixture, was isolated and purified by a method employing ion exchange chromatography followed by carbon column chromatography and crystallization (10).

Viomycidine was assigned the formula $C_6H_{10}N_4O_2$ on the basis of analysis of its monohydrochloride salt. No N-methyl, O-methyl, C-methyl, or primary amino groups are present. The monohydrochloride salt is a colorless, crystalline solid that melts with decomposition

at 200-208°. Viomycidine is optically active; the specific rotation varies with the pH of the solution (10).

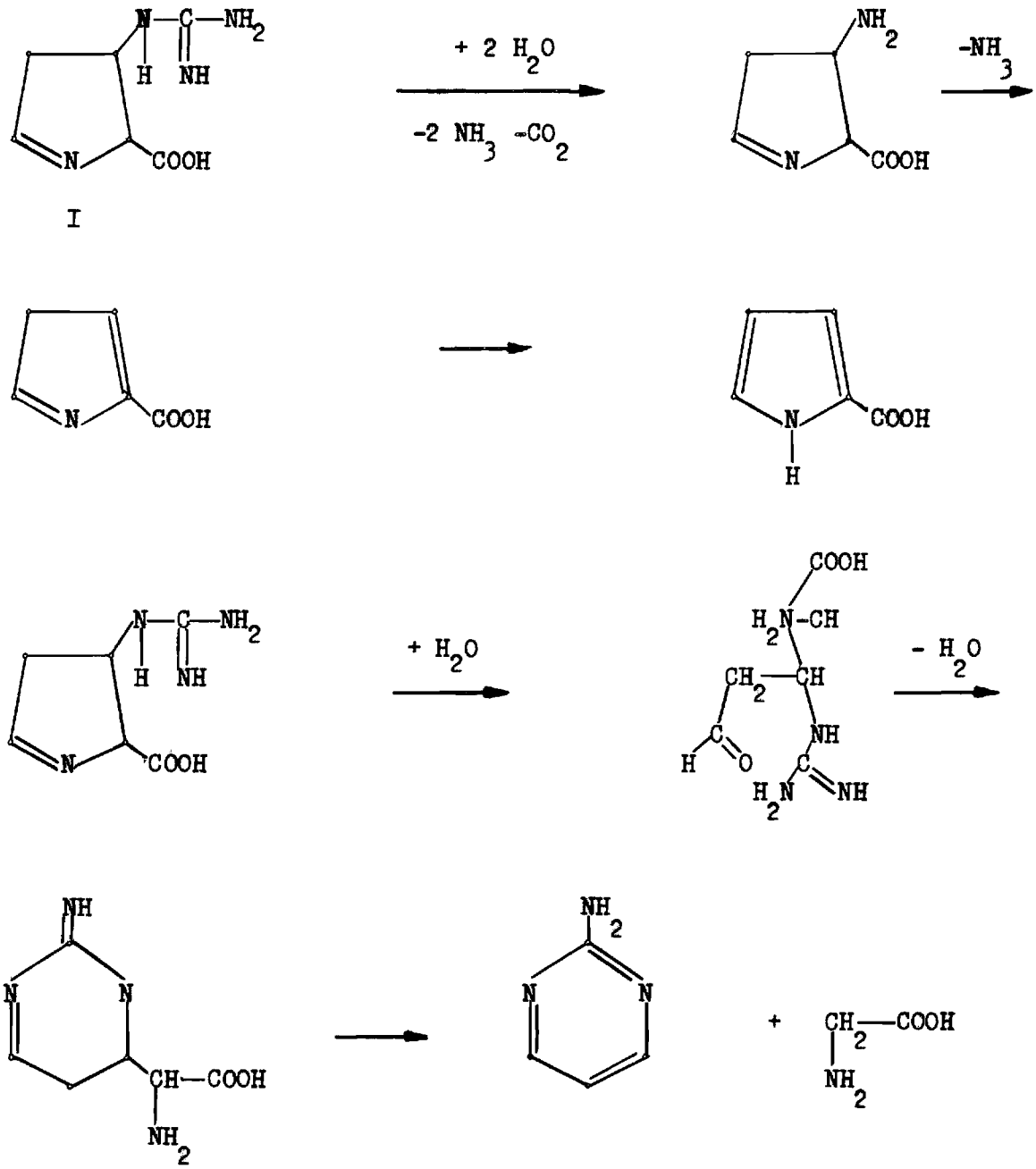
Viomycidine has pK_a values of 2.8, 5.87 and 13.4 in 66 per cent N,N-dimethylformamide and 5.50 and 12.6 in water. The pK_a of the most acidic center was too low to be determined in water. The pK_a values indicate the presence of a carboxyl group, a weakly basic group, and a guanidine group, respectively (10). Since the group whose pK_a value is 5.50 has a differential ultraviolet spectrum, the weakly basic group appears to be a tertiary amino group (11). The presence of the guanidine group also is indicated by the positive Sakaguchi test. Only monosubstituted guanidines give this test.

It will be seen from the formula that viomycidine contains four rings and/or double bonds. The carboxylic acid and guanidine groups account for two double bonds. Viomycidine absorbed one mole of hydrogen, although the reduction product has not been isolated. This indicated that viomycidine has a cyclic structure (10,11).

A Δ^1-pyrroline structure was proposed for viomycidine (11). This structure would account for the ring, the double bond and the tertiary amine (imine) group of viomycidine. This structure was confirmed by the fact that viomycidine gave a yellow colored solution when treated with o-aminobenzadehyde reagent, typical of Δ^1-pyrrolines (11).

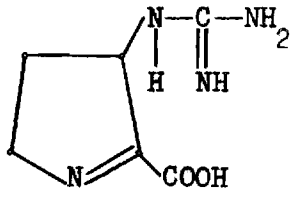
The structure 4-guanidino-Δ^1-pyrroline-5-carboxylic acid (I) was proposed for viomycidine on the basis of alkaline hydrolyses. When treated with hot aqueous barium hydroxide, viomycidine gave three moles of volatile base (ammonia), one mole of carbon dioxide, and 0.25 mole of pyrrole-2-carboxylic acid.

When treated with hot concentrated sodium hydroxide, viomycidine gave 2-aminopyrimidine and glycine (11). The following reaction pathways were proposed for these alkaline hydrolyses.



I

Nuclear magnetic resonance spectra of viomycidine hydrochloride in deuterium oxide and in trifluoroacetic acid are not satisfied by the structure 4-guanidino-Δ'-pyrroline-5-carboxylic acid (I). The spectra are satisfied, however, by the structure 3-guanidino-Δ'-pyrroline-2-carboxylic acid (II). Isomerization of II to I in alkaline solution was assumed to be facile (11).



II

In addition to being treated with boiling 6 N mineral acid, viomycin was subjected to milder hydrolytic conditions. It was hoped that conditions for obtaining higher yields of viomycidine and for partially degrading viomycin would be found. None of the hydrolytic conditions studied gave higher yields of viomycidine than boiling 6 N mineral acid hydrolysis of viomycin. However, some of these milder hydrolytic conditions did appear to cleave selectively certain peptide bonds. Thus, when viomycin was treated with 1 N hydrochloric acid at 95°, urea, serine, and β-lysine were released within five hours (4).

The most fruitful of the hydrolytic conditions studied was 0.1 N hydrochloric acid at 95°. Under these conditions, one mole of urea was released in six hours. Only traces of carbon dioxide, ammonia, and amino acids were present in the 0.1 N hydrochloric acid hydrolysate after 23 hours. The other product of this hydrolysis, desureaviomycin, was isolated and its properties investigated (4).

Desureaviomycin gave a positive ninhydrin test. When treated with boiling 6 N hydrochloric acid it gave all the compounds present in the 6 N acid hydrolysate of viomycin except urea. It formed a trihydrochloride salt, as shown by titration with silver nitrate (4).

Dilute alkaline hydrolysis of viomycin gave urea, serine, β-lysine, and 2,3-diaminopropionic acid. The absence of viomycidine from alkaline hydrolysates is anticipated, since it is degraded by dilute alkali (4).

Viomycin was oxidized by neutral permanganate. In the oxidation viomycin rapidly reduced four equivalents of permanganate with destruction of its ultraviolet chromophore. A fifth equivalent of permanganate also was reduced rapidly. No fragmentation of viomycin to amino acids, urea, carbon dioxide, volatile acid, or oxalic acid took place in the four-equivalent oxidation. No carboxyl groups were produced in the oxidation. The five-equivalent oxidation released some urea and a carboxyl group was formed. Paper chromatography indicated that the 6 N acid hydrolysate of the five-equivalent oxidation product of viomycin contained the same compounds as the 6 N acid hydrolysate of viomycin itself (4).

The Sanger procedure was applied to viomycin in order to determine which primary amino groups of viomycin are free and which ones are used in peptide bonds. Van Slyke amino nitrogen values indicated that viomycin has two primary amino groups while four more are released on 6 N acid hydrolysis. The 2,4-dinitrophenyl derivative of viomycin is a bright yellow solid that decomposes between 250 and 260°. When treated with 6 N hydrochloric acid at 95°, in which it is slightly

soluble, 2,4-dinitrophenylviomycin gave urea, serine, 2,3-diaminopropionic acid, and viomycidine. The absence of β-lysine from this hydrolysate indicated that at least one of the amino groups of the β-lysyl residue of viomycin was free. However, the 2,4-dinitrophenyl derivative of β-lysine presumed to be released by hydrolysis was not identified (4).

EXPERIMENTAL

Apparatus and Techniques

Ion exchange resins were used and regenerated as described previously (10). The following abbreviations are used: IR-45 for Amberlite anion exchange resin 45; IR-400 for Amberlite anion exchange resin 400; and IR-50 for Amberlite cation exchange resin 50. Ion exchange resins were used both in batch and in column operations. When resins were used in batches, the sample was eluted with a volume of solvent about four times the volume of the settled resin. When resins were used in columns, the sample was eluted with a volume of solvent at least twice the volume of the column.

Apparatus and techniques used in paper chromatography include those previously described (10,12,13). In addition, strips of chromatography paper about 12 x 40 cm. in size supported by glass racks were used. The two most useful solvent systems were t-butyl alcohol-acetic acid-water, 2:1:1 (v/v) and 0.5 M sodium monohydrogen phosphate-1.0 M potassium dihydrogen phosphate (pH 6.0). These two solvent systems are called BAW and phosphate buffer, respectively. Spray reagents most frequently used were ninhydrin, Weber, and p-dimethyl-aminobenzaldehyde reagents. The ninhydrin reagent consisted of 0.2 per cent ninhydrin in 50 per cent by volume pyridine. The reagent is stable indefinitely when kept in a refrigerator. After being sprayed, the chromatogram was allowed to dry and then was heated over a hot plate. 2,3-Diaminopropionic acid gave an orange to pink colored spot at room

temperature. Other amino acids gave distinctively colored spots when the chromatogram was heated. The Weber reagent was prepared as previously described (12,14). The reagent was prepared immediately before each use. Guanidines gave orange to blue colored spots that often faded rapidly. The p-dimethylaminobenzaldehyde reagent was prepared as previously described (12,15). The reagent was prepared immediately before each use. Urea and substituted ureas gave yellow colored spots. 2,3-Diaminopropionic acid and viomycin gave slowly developing yellow spots.

Ninhydrin and Nessler's reagents were used routinely for color tests. The ninhydrin reagent used was the same as the spray reagent used in paper chromatography. One drop of the solution to be tested and about 0.5 ml. of the reagent were heated in a small test tube on the steam bath. A pink to blue color that develops within ten minutes is a positive test. The Nessler's reagent was prepared and used to detect ammonia as described previously (10).

Anhydrous hydrazine used in hydrazinolysis studies was distilled as described in Organic Syntheses (16). The distilled anhydrous hydrazine was kept in sealed glass ampules until immediately before use.

A Beckman model DK-1 recording spectrophotometer was used to obtain ultraviolet spectra. A Perkin-Elmer model 137 recording spectrophotometer was used to obtain infrared spectra. Potassium bromide pellets were used in infrared spectroscopy. A Varian model A-60 spectrometer was used to obtain nuclear magnetic resonance spectra. Tetramethylsilane was the internal standard when trifluoroacetic acid was the solvent. Sodium 2,2-dimethyl-2-silapentane-5-sulfonate was the internal standard when water or deuterium oxide was the solvent.

All melting points were determined on a Kofler micro hot stage. Corrected values of melting points are given. All values of specific rotation were determined at the sodium D line with a Bellingham and Stanley, Ltd. polarimeter.

Analyses were performed by Huffman Microanalytical Laboratories, Wheatridge, Colorado.

Viomycin

Preparation and Properties of Viomycin Sesquisulfate

A solution of 17.3 g. of viomycin sulfate, as received from Parke, Davis & Company, in a few milliliters of water was treated with 200 ml. of IR-45 in the hydroxyl form. The pH of the solution rose to 8. The solution was applied to a column of 230 ml. of IR-45 in the sulfate form and the column was eluted with water until the effluent gave a negative ninhydrin test. Evaporation of the water by lyophilization gave viomycin sesquisulfate.

An analytical sample of viomycin sesquisulfate was prepared by repeated precipitations from aqueous methanol. Viomycin sesquisulfate was dissolved in a few milliliters of water and methanol was added until slight turbidity resulted. The turbidity was removed by addition of a few drops of water. The solution was then cooled in an ice bath to give a gummy precipitate. The mixture was removed from the ice bath and the precipitate was triturated by magnetic stirring, yielding a flocculent white powder. The mixture was then returned to the ice bath. The precipitate was collected by filtration and was washed with cold aqueous methanol. The precipitation was repeated three times. The white powder was dried *in vacuo* in a desiccator over calcium chloride at room temperature to constant weight.

Anal. $C_{25}H_{44}N_{12}O_{11} \cdot 3/2\ H_2SO_4$
(835.8)

Calc'd: C, 35.93; H, 5.67; N, 20.11; S, 5.75

$C_{25}H_{45}N_{13}O_{11} \cdot 3/2\ H_2SO_4$
(850.8)

Calc'd: C, 35.29; H, 5.69; N, 21.40; S, 5.66

Found: C, 35.01; H, 6.19; N, 21.01; S, 5.73

The analytical sample was dried to constant weight in vacuo at 80° prior to all analyses; 7.8 per cent was lost during 48 hours. Van Slyke primary amino nitrogen values were 2.14 per cent after 2.5 minutes, 3.47 per cent after 15 minutes, and 3.78 per cent after 60 minutes.

The specific rotation of hydrated viomycin sesquisulfate was -33.5° (five per cent in water). The infrared spectrum of hydrated viomycin sesquisulfate had strong bands at 3.0, 6.0, and 9.0μ; medium bands at 3.2, 3.4, 6.7, and 8.2μ; and weak bands at 4.8, 7.4, and 7.6μ. All bands were broad. A solution of hydrated viomycin sesquisulfate in water had an absorption peak at 268 mμ(ε, 24,600). A solution of hydrated viomycin sesquisulfate in 0.1 N hydrochloric acid had an absorption peak at 267 mμ(ε, 27,200). The nuclear magnetic resonance spectrum of a 40 per cent solution of viomycin sesquisulfate in deuterium oxide was recorded (Figure 1). The size of the water peak was reduced by five lyophizations of solutions of viomycin sesquisulfate in deuterium oxide. The spectrum was traced on paper, and peaks were cut out and weighed. Assuming that the weight of the peak centered at 1.93τ corresponded to one proton, the weight of the peak centered at 6.00τ corresponded to 7.4 protons, that of the two peaks centered at 6.90

and 7.17 τ corresponded to 6.8 protons, and that of the peak at 8.22 τ corresponded to 6.5 protons.

A few drops of Fehling's reagent (17) was added to a solution of about 10 mg. of viomycin sulfate in a milliliter of water. A deep violet solution resulted. The solution was heated for ten minutes on a steam bath and allowed to stand for a day. No precipitate formed.

To a solution of 16.2 mg. (0.02 millimoles) of viomycin sulfate in 5 ml. of water was added a solution of 2.7 mg. (0.03 millimoles) of thiosemicarbazide in 4 ml. of water. The pH of the resulting solution was about 4.5. The solution was allowed to stand at room temperature overnight and then was diluted to 100 ml. with water. Five milliliters of this solution was diluted to 100 ml. with water for ultraviolet spectroscopy. The ultraviolet spectrum was identical to that of viomycin over the region 260 to 350 mμ.

The o-aminobenzaldehyde reagent was prepared as by Jakoby and Fredricks (18). The reagent contained about 40 mg. of o-aminobenzaldehyde in 10 ml. water. About 5 mg. of viomycin, 0.5 ml. of reagent, and 0.5 ml. of 0.025 N hydrochloric acid were mixed in a small test tube and heated on a steam bath for about two minutes. No color developed.

Acid Hydrolysis of Viomycin

A solution of 200 mg. of viomycin sulfate in 2 ml. of 6 N hydrochloric acid was heated under reflux on a steam bath for 16 hours. This hydrolysate was studied by paper chromatography using several developing solvents. Solvents used included t-butyl alcohol-acetic acid-water of several compositions; t-butyl alcohol-pyridine-water of

several compositions; 2,6-lutidine-water, 13:7 (v/v); ethanol-acetic acid-water, 16:1:4 (v/v); and ethanol-concentrated ammonium hydroxide-water, 16:1:4 (v/v). The chromatograms were sprayed with ninhydrin reagent. The best overall separation of spots was obtained with t-butyl alcohol-acetic acid-water, 2:1:1 (v/v) (BAW). Chromatograms spotted with the hydrolysate, serine, β-lysine, viomycidine, and 2,3-diamino-propionic acid were developed with BAW and sprayed with ninhydrin and Weber reagents. The results are given in Table 3.

A solution of 290 mg. of viomycin sesquisulfate in 10 ml. of 6 N hydrochloric acid was heated under reflux on a steam bath in a two-necked 50-ml. round-bottomed flask for 16 hours. Nitrogen was bubbled through 5 N sodium hydroxide and then admitted through one neck of the flask to aerate the solution. The reflux condenser was placed in the other neck of the flask. The nitrogen passed out the top of the reflux condenser and passed through calcium chloride and Drierite drying tubes. The gas was then passed through a tube containing Ascarite. The Ascarite gained 30.5 mg. In a blank determination the Ascarite gained 8.1 mg. The determination was repeated as above, using 272 mg. of viomycin sesquisulfate. The hydrolysis was stopped after six hours. The Ascarite gained 17.3 mg. In a blank determination for six hours the Ascarite gained 4.6 mg.

A solution of 100 mg. of viomycin sulfate in one milliliter of 6 N hydrochloric acid was allowed to stand at room temperature for two weeks. Spots were applied to paper for chromatography at zero time and after 1, 2, 3, 4, 8, 10, 11, and 14 days. After development with BAW, papergrams were sprayed with ninhydrin, Weber and p-dimethyl-aminobenzaldehyde reagents. Each of the spots applied to the

chromatograms had some material that remained at the origin and gave colors with each of the spray reagents. The chromatogram sprayed with ninhydrin reagent had spots whose R_F values, colors, and intensities of color *versus* time were as follows: 0.38-0.41, grey-blue, faint after one day and increasing until the fourth day; 0.48-0.52, purple, very faint after one day and increasing until the tenth day; and 0.19-0.20, orange at room temperature, faint after eleven days and increasing until the fourteenth day. The chromatogram sprayed with *p*-dimethylamino-benzaldehyde had a yellow spot whose R_F value was 0.70-0.72. This spot was present after one day of hydrolysis and did not increase in intensity thereafter. The chromatogram sprayed with Weber reagent had a pink streak from the origin after one day of hydrolysis.

Hydrogenation of Viomycin

One gram of viomycin sulfate was dissolved in 86 ml. of 58 per cent by volume acetic acid. To this solution was added 210 mg. of five per cent platinum on carbon (Baker and Company, Newark, New Jersey). The mixture was shaken in a pressure bottle under hydrogen at 40 lb./in.^{2} for three days. The mixture was filtered through a cake of Celite on a sintered-glass funnel. The filtrate was concentrated to about five milliliters by evaporation *in vacuo* and treated with IR-45 in the hydroxyl form until the pH of the solution rose to 6. The solution was applied to a column of 10 ml. of IR-45 in the sulfate form, and the column was eluted with water until the effluent gave a negative test with ninhydrin reagent. Evaporation of the water by lyophilization gave 980 mg. of white powder.

This material had an absorption peak at 268 mμ (ϵ, 19,500) in

water. Microbiological assay of this material showed that it had 70 per cent of the activity of viomycin sesquisulfate (19). The nuclear magnetic resonance spectrum of a 20 per cent solution of this material in deuterium oxide was essentially identical with that of viomycin sulfate.

Ozonolysis of Viomycin

Ozone was passed through a solution of 200 mg. of viomycin sulfate in 100 ml. of 20 per cent by volume acetic acid for an hour. The solution was then aerated with nitrogen until the exit gases gave a negative test with moist starch-potassium iodide paper. A drop of concentrated sulfuric acid and 1 ml. of 30 per cent hydrogen peroxide were added to the solution. All the above operations were performed at ice-water bath temperatures.

A 1-ml. aliquot of the solution was diluted to 100 ml. and the ultraviolet spectrum of the dilute solution was obtained. The peak of viomycin at 268 mμ was absent; only end absorption remained.

The above procedure was repeated using a solution of 6.06 g. of viomycin in 60 ml. of 16.7 per cent by volume acetic acid. Ozone was passed through the solution for two hours. After the solution was aerated with nitrogen, a milliliter of concentrated sulfuric acid and 5 ml. of 30 per cent hydrogen peroxide were added. Paper chromatograms (BAW) of the solution had one ninhydrin-positive spot at the origin. To this solution was added an equal volume of concentrated hydrochloric acid. The resulting solution was heated overnight under reflux on a steam bath. The hydrolysate was treated with IR-45 in the hydroxyl form in batches until the pH rose to 6. The solution was then concentrated

to about 20 ml. and paper chromatograms (BAW) prepared. Chromatograms sprayed with ninhydrin reagent had spots whose R_F values and colors were as follows: 0.48, purple; 0.38, grey-blue; 0.32, pink; 0.29, orange at room temperature; and 0.16, green. Chromatograms sprayed with Weber reagent had spots whose R_F values and colors were as follows: 0.70, orange; 0.32, pink; and 0.16, pink.

The hydrolysate was applied to a column of 96 ml. of IR-400 in the hydroxyl form. The column was eluted with 300 ml. of water. The water eluate was concentrated to about 20 ml. and paper chromatograms (BAW) were prepared. Chromatograms sprayed with ninhydrin reagent had spots whose R_F values and colors were 0.32, pink and 0.16, green. Chromatograms sprayed with Weber reagent had spots whose R_F values and colors were as follows: 0.70, orange; 0.32, pink; and 0.16, pink.

The IR-400 column was eluted with 1.2 N hydrochloric acid until the effluent gave a negative ninhydrin test. The effluent was neutralized by being passed down a column of 80 ml. of IR-45 in the hydroxyl form. The solution was concentrated to about 10 ml. by evaporation, and paper chromatograms (BAW) were prepared. Chromatograms sprayed with ninhydrin reagent had spots whose R_F values and colors were as follows: 0.46, purple; 0.36, grey-blue; 0.29, pink at room temperature; 0.17, green. Chromatograms sprayed with Weber reagent had one spot whose R_F value was 0.16 and whose color was pink.

DNP-Viomycin

All 2,4-dinitrophenyl derivatives were prepared by reaction of the amino acid or peptide with excess 2,4-dinitrophenylfluorobenzene in aqueous ethanolic sodium bicarbonate solution (20,21). The reaction

mixture was shaken two hours and the ethanol was removed by evaporation *in vacuo* at 40-60°. If the N-2,4-dinitrophenyl (DNP) derivative was insoluble in the resulting aqueous bicarbonate solution, it was collected by filtration and washed with copious amounts of 1 N hydrochloric acid, ethanol and ether. Bis-DNP-viomycin was isolated in this way. If the DNP derivative was soluble in aqueous bicarbonate solution, the solution was extracted three times with equal volumes of ether and the ether solutions discarded. Then the DNP derivative was precipitated by acidification of the aqueous layer with concentrated hydrochloric acid. DNP-L-serine, DNP-β-alanine and DNP-DL-aspartic acid were isolated in this way.

DNP-L-Serine was prepared and isolated as by Rao and Sober (22). The yield, after recrystallization from benzene-ethyl acetate, was 32 per cent of theoretical. The melting point was 175-176° (lit. (22) 173-174°).

DNP-β-Alanine was prepared as by Rao and Sober (22). The solid that precipitated on acidification of the aqueous layer was dissolved in saturated sodium bicarbonate solution. The solution was acidified slowly with 1 N hydrochloric acid to give crystalline material. The yield, after crystallization from hot aqueous ethanol, was 31 per cent of theoretical. The melting point was 145-146° (lit. (22) 145-146°).

DNP-DL-Aspartic acid was prepared and isolated as by Porter and Sanger (23). The yield, after recrystallization from benzene-acetone, was 80 per cent of theoretical. The melting point was 190° (lit. (23) 190°).

Bis-DNP-β-lysine was prepared as follows: 282 mg. of crystalline

β-lysine di-p-hydroxyazobenzene-p'-sulfonate was dissolved in hot water and applied to a column of 15 ml. of IR-45 in the hydroxyl form. The column was eluted until the effluent gave a negative ninhydrin test. The effluent was concentrated by evaporation *in vacuo* to about 20 ml. One gram of sodium bicarbonate and a solution of 1 g. of 2,4-dinitrofluorobenzene in 20 ml. of absolute ethanol were added. The reaction mixture was allowed to stand for two hours with occasional swirling. The ethanol was removed by evaporation *in vacuo* and the mixture was acidified with concentrated hydrochloric acid. The mixture was filtered and the yellow residue was washed well with 1 N hydrochloric acid, ethanol, and ether. The filtrate and washings were discarded. The crude bis-DNP-β-lysine was dried overnight in a desiccator over calcium chloride. The yield was 154 mg. (80 per cent of theoretical) of material melting at 193-196°. This material was chromatographed on silicic acid. One major band was eluted rapidly with ethyl acetate. On evaporation this band gave 136 mg. (71 per cent of theoretical) of bright yellow needles melting at 200-201° (lit. (24) 196-197°).

The ultraviolet spectra of DNP-L-serine, DNP-β-alanine, DNP-DL-aspartic acid and bis-DNP-β-lysine solutions in one per cent N,N-dimethylformamide were obtained. The required solutions were prepared by dissolving 0.96 to 1.23 mg. of the DNP derivative in one milliliter of redistilled N,N-dimethylformamide and diluting the solution to 100 ml. with water. In addition the following ultraviolet spectra were obtained: DNP-L-serine and DNP-DL-aspartic acid in redistilled glacial acetic acid; DNP-β-alanine and DNP-DL-aspartic acid in 1 N sodium hydroxide; and DNP-L-serine in four per cent sodium bicarbonate.

The above spectra were obtained with a Beckman model DK recording spectrophotometer. Table 5 gives the extinction coefficients at maximum absorption (339-361 mμ) calculated from these spectra. Available literature values are also given in the table.

Bis-DNP-viomycin was prepared as by Mason (4). In a typical preparation, 500 mg. of viomycin sulfate gave 583 mg. of bis-DNP-viomycin. Bis-DNP-viomycin blackened when heated above 220°. Mason reported that it decomposed at 250-260°.

The ultraviolet spectrum of 2.67 mg. of bis-DNP-viomycin in 100 ml. of one per cent N,N-dimethylformamide solution was obtained with a Beckman model DK recording spectrophotometer. The spectra of this solution and of a solution of bis-DNP-β-lysine in one per cent N,N-dimethylformamide also were obtained over the region 350-365 mμ with a Beckman DU quartz spectrophotometer. The absorption at 359 mμ was 0.604.

Bis-DNP-viomycin was hydrolyzed in concentrated (ca. 12 N) hydrochloric acid in a pressure bottle on a steam bath; in dioxane-concentrated hydrochloric acid, 1:1 (v/v) under reflux on a steam bath; and in boiling 6 N hydrochloric acid under reflux. Paper chromatography was used to follow the hydrolyses. In each case the hydrolysis appeared to be complete in six hours.

One gram of bis-DNP-viomycin and 50 ml. of concentrated hydrochloric acid were placed in a pressure bottle and the bottle was stoppered. The bottle was heated on a steam bath with occasional shaking for 20 hours. The bis-DNP-viomycin dissolved, the solution developed a light orange color, and a fine, bright yellow precipitate

formed on the wall of the vessel. The mixture was concentrated to about ten milliliters by evaporation in vacuo and was filtered. The residue and the bright yellow solid scraped from the bottle were combined.

Results of paper chromatography (BAW) of the filtrate are summerized in Table 4. The residue was washed with ethyl acetate. Most of the residue dissolved; the material that did not dissolve was discarded. Paper chromatograms of the ethyl acetate solution were prepared using BAW and phosphate buffer as developing solvents. The chromatogram developed using BAW had a single yellow spot whose R_F value was 0.89. The chromatogram developed using phosphate buffer had a single yellow spot at the origin. The ethyl acetate solution was concentrated by evaporation and applied to a silicic acid column, measuring 4.2 x 26 cm. One major band was eluted rapidly with ethyl acetate. On evaporation, 301 mg. of bright yellow solid, m.p. 197-198°, was obtained. The melting point was not depressed by admixture with synthetic bis-DNP-β-lysine.

Two grams of bis-DNP-viomycin and 100 ml. of 6 N hydrochloric acid were boiled under reflux on a sand bath for six hours. The bis-DNP-viomycin did not dissolve, the solution developed a dark orange-brown color, and black, tarry material formed on the wall of the vessel. The mixture was filtered and paper chromatograms of the filtrate prepared. An unsprayed chromatogram developed in BAW had yellow spots whose R_F values were 0.89 and 0.67. A chromatogram that was developed with BAW and sprayed with ninhydrin had spots whose R_F values and colors were as follows: 0.89, yellow; 0.67, purple; 0.48, purple; 0.32, pink; 0.29, orange at room temperature; and 0.17, green.

A chromatogram that was developed with BAW and sprayed with Weber reagent had a pink spot whose R_F value was 0.32. An unsprayed chromatogram developed in phosphate buffer had yellow spots whose R_F values were 0.54 and 0.72. The spot whose R_F value was 0.54 disappeared when the chromatogram was exposed to hydrogen chloride vapors.

The residue was washed with ethyl acetate and the ethyl acetate-insoluble material was discarded. An unsprayed chromatogram developed in phosphate buffer had yellow spots whose R_F values were 0.54 and 0.00. The spot whose R_F value was 0.54 disappeared when the chromatogram was exposed to vapors of hydrogen chloride.

Hydrolysis of bis-DNP-viomycin with dioxane-concentrated hydrochloric acid gave results similar to those with 6 N hydrochloric acid. Bis-DNP-viomycin dissolved in the solution, but black, tarry material formed on the wall of the flask. The mixture was filtered. Paper chromatograms of the filtrate and ethyl acetate washings of the residue were similar to those of the 6 N hydrochloric acid hydrolysate and ethyl acetate washings of the residue.

Hydrazinolysis of Viomycin

One hundred milligrams of viomycin sulfate and about 1.5 ml. of anhydrous hydrazine were placed in a thick-walled glass tube. The solution was frozen and the tube sealed. The tube was allowed to warm to room temperature and then was heated in an oil bath at 110-130° for 18 hours. The sealed tube was cooled, opened, and the solution transferred to a 10-ml. beaker. Hydrazine was removed by evaporation *in vacuo* in a desiccator over concentrated sulfuric acid. The resulting solid was dissolved in a milliliter of water and the pH of the resulting

solution adjusted to 7 by addition of a few drops of phosphate buffer. A milliliter of benzaldehyde was added and the mixture was allowed to stand overnight. Excess benzaldehyde was removed, and the aqueous layer was extracted with two milliliters of ether. Paper chromatograms (BAW) of the aqueous layer had only very faint spots after being sprayed with ninhydrin. None of these faint spots had R_F values similar to those recorded in Table 3.

Desureaviomycin

Preparation and Properties

Desureaviomycin hydrochloride was prepared and isolated by a procedure based on that of Mason. In a typical preparation, a solution of 3.76 g. of viomycin sulfate in 50 ml. of 0.1 N hydrochloric acid was heated under reflux on a steam bath for six hours. The hydrolysate was neutralized with IR-45 in the hydroxyl form in a batch operation. The solution was then applied to a column of 7 ml. of IR-45 in the chloride form. The column was eluted with water until the effluent gave a negative test with ninhydrin reagent. The solution was then evaporated to dryness *in vacuo*. After being dried overnight *in vacuo* in a desiccator over calcium chloride, the tan solid weighed 3.57 g. This solid was triturated with, and washed by filtration with two 10-ml. portions of warm absolute ethanol. The residue, desureaviomycin trihydrochloride, weighed 3.05 g. after air-drying. The ethanol solution was concentrated by evaporation *in vacuo* to a few milliliters, and paper chromatograms of it were developed with BAW. The chromatograms were sprayed with ninhydrin, Weber, and p-dimethylaminobenzaldehyde reagents. The chromatograms sprayed with ninhydrin and Weber reagents had no spots.

The chromatogram sprayed with p-dimethylaminobenzaldehyde reagent had one yellow spot whose R_F value was 0.70.

Desureaviomycin trihydrochloride could not be crystallized from aqueous ethanol or aqueous acetone. Instead, heavy syrups formed. Three grams of desureaviomycin trihydrochloride was dissolved in a few milliliters of water and treated with IR-45 in the hydroxyl form in batches until the pH rose to about 8. The solution was then passed down a column of 20 ml. of IR-45 in the sulfate form in a 25-ml. buret. Desureaviomycin sesquisulfate also gave heavy syrups on attempted crystallization from aqueous ethanol or aqueous acetone.

A sample was prepared for analysis as follows: three grams of desureaviomycin sesquisulfate was dissolved in 6 ml. of cold water and 4 ml. of cold ethanol was added. The resulting heavy syrup was separated by centrifugation and the supernatant liquid was removed. The precipitation was repeated three times. The final syrup was dissolved in a few milliliters of water and dried by lyophilization to give 1.62 g. of light tan powder.

Anal. $C_{24}H_{40}N_{10}O_{10} \cdot 3/2\ H_2SO_4$
(775.8)

Calc'd: C, 37.14; H, 5.58; N, 18.05; S, 6.20

Found: C, 37.21; H, 5.50; N, 18.67; S, 5.63

The analytical sample was dried *in vacuo* at 80° to constant weight prior to all analyses; 10.23 per cent was lost during 144 hours. Van Slyke primary amino nitrogen values were 3.63 per cent after 2.5 minutes, 4.15 per cent after 15 minutes, and 4.77 per cent after 60 minutes.

Desureaviomycin trihydrochloride had specific rotation of -32° (5.04 per cent in water). The infrared spectrum of desureaviomycin sesquisulfate had strong bands at 2.9, 6.0, and 9.0μ; medium bands at 3.2, 3.4, and 6.6μ; and weak bands at 4.8, and 7.4μ. A solution of desureaviomycin sesquisulfate in 0.1 N hydrochloric acid had an absorption peak at 267 mμ (ε, 5,500). The nuclear magnetic resonance spectrum of a 20 per cent solution of desureaviomycin sesquisulfate in deuterium oxide was obtained (Figure 2). The size of the water peak was reduced by repeated lyophilization of solutions of desureaviomycin sesquisulfate in deuterium oxide. The spectrum was traced on paper, and peaks were cut out and weighed. Assuming that the weight of the peak centered at 1.92τ corresponded to one proton, the weight of the peak centered at 5.97τ corresponded to 8.1 protons, that of the peaks centered at 6.87 and 7.12τ corresponded to 6.2 protons, and that of the peak centered at 8.15τ corresponded to 6.5 protons.

DNP-Desureaviomycin

The DNP derivative of desureaviomycin was prepared by essentially the same procedure as was bis-DNP-viomycin. In a typical preparation, one gram of sodium bicarbonate and a solution of one gram of 2,4-dinitrofluorobenzene in 20 ml. of absolute ethanol were added to a solution of 201 mg. of desureaviomycin sesquisulfate in 20 ml. of water. The reaction mixture was allowed to stand for two hours with frequent swirling. Ethanol was removed by evaporation in vacuo and the solution was acidified with concentrated hydrochloric acid. The mixture was filtered and the residue was washed well with 1 N hydrochloric acid, ethanol, and ether. After being dried overnight in vacuo at 80°, the

bright yellow powder weighed 254 mg. Bis-DNP-desureaviomycin darkened when heated above 200°, but did not melt below 300°. The ultraviolet spectrum of a solution of 1.68 mg. of bis-DNP-desureaviomycin in 100 ml. of one per cent N,N-dimethylformamide was obtained. The absorption at 357 mμ was 0.402.

Twenty milliliters of concentrated (ca. 12 N) hydrochloric acid and 250 mg. of bis-DNP-desureaviomycin were placed in a pressure bottle. The bottle was stoppered and heated on a steam bath with occasional shaking for 20 hours. The bis-DNP-desureaviomycin dissolved, the solution became orange in color, and a bright yellow precipitate formed. The reaction mixture was filtered. Paper chromatograms of the filtrate developed with BAW and sprayed with ninhydrin had spots whose R_F values and colors were as follows: 0.17, green; 0.29, orange at room temperature; 0.32, pink; 0.48, purple; and 0.87, faint yellow before and after spraying. The residue and the yellow solid scraped from the bottle were combined. This material was washed with ethyl acetate and ethyl acetate-insoluble material was discarded. A paper chromatogram of the ethyl acetate solution was developed with phosphate buffer as solvent. The unsprayed chromatogram had one intense yellow spot at the origin. The ethyl acetate solution was applied to a silicic acid column measuring 2.3 x 23.5 cm. One major band was eluted rapidly with ethyl acetate. On evaporation, 27 mg. of bright yellow solid, m.p. 198-199°, was obtained. The melting point was not depressed on admixture with synthetic bis-DNP-β-lysine.

Hydrazinolysis of Desureaviomycin

One hundred milligrams of desureaviomycin sesquisulfate and about

1.5 ml. of anhydrous hydrazine were placed in a thick-walled glass tube and the tube was sealed. Desureaviomycin sesquisulfate dissolved in the hydrazine and the solution was heated in the sealed tube in an oil bath at 120° for 18 hours. The sealed tube was cooled, opened, and the solution transferred to a 10-ml. beaker. The hydrazine was removed by evaporation in vacuo in a desiccator over concentrated sulfuric acid. The resulting solid was dissolved in a milliliter of water and the pH of the solution adjusted to 7 with a few drops of phosphate buffer. A milliliter of benzaldehyde was added and the mixture allowed to stand overnight. The excess benzaldehyde was removed and the aqueous layer was extracted with two milliliters of ether. Paper chromatograms of the solution were developed with BAW as solvent. A chromatogram sprayed with ninhydrin reagent had one purple spot whose R_F value was 0.48. In addition, there were some very faint spots of low R_F value and a faint streak from the origin. A chromatogram sprayed with Weber reagent had no spots.

Viomycinic Acid

Preparation and Properties

Eighty milligrams of desureaviomycin was dissolved in a milliliter of water and 0.2 ml. of carboxypeptidase suspension (California Corporation for Biochemical Research) was added. The pH of the solution was adjusted to 7.5 with a few drops of ten per cent potassium carbonate and the mixture was allowed to stand at room temperature. Spots were applied to paper for chromatography at zero time and after 1 hour, 2 hours, 4 hours, 1 day, 2 days, 11 days, 14 days, and 20 days. The chromatograms were developed with BAW and sprayed with ninhydrin reagent.

The chromatograms had purple spots at the origin for each application. A purple spot of R_F value 0.48 appeared at the application made after one day of hydrolysis. This spot increased in size and intensity until the second day. No other spots appeared on the chromatograms.

The enzymatic hydrolysis of desureaviomycin with carboxypeptidase was repeated. Five grams of desureaviomycin sulfate was dissolved in 15 ml. of water and 0.5 ml. of carboxypeptidase suspension was added. The pH of the solution was adjusted to 7.5 by addition of a little IR-400 resin in the hydroxyl form. The mixture was allowed to stand for two days at room temperature. The mixture was filtered and applied to a column of 475 ml. of IR-50 in the hydrogen form. The column was eluted with 1500 ml. of water. Evaporation of the water gave 1.63 g. of a light tan solid. Paper chromatograms developed with BAW and sprayed with ninhydrin reagent had purple spots whose R_F values were 0.48 and 0.00.

The IR-50 column was then eluted with 900 ml. of 1 N sulfuric acid. The effluent was neutralized immediately with IR-45 in the hydroxyl form in a batchwise operation. The IR-45 was eluted with water and the effluent was applied to a column of 20 ml. of IR-45 in the sulfate form. This column was eluted with water until the effluent gave a negative test with ninhydrin reagent. Evaporation of the water gave 3.29 g. of a light tan powder. A paper chromatogram of this material developed with BAW and sprayed with ninhydrin reagent had a purple spot at the origin. The sample was dried to constant weight at 80° *in vacuo*; the loss was 10.79 per cent in 36 hours. Van Slyke primary amino nitrogen values were 5.08 per cent after 2.6 minutes, 5.56 per cent

after 15 minutes, 5.75 per cent after 60 minutes, and 6.35 per cent after four hours.

Anal. $C_{21}H_{35}N_9O_8 \cdot 3/2\ H_2SO_4$
(688.7)

Calc'd: C, 36.62; H, 5.56; N, 18.30; S, 6.98

$C_{21}H_{36}N_{10}O_8 \cdot 3/2\ H_2SO_4$
(703.7)

Calc'd: C, 35.84; H, 5.59; N, 19.90; S, 6.84

Found: C, 35.68; H, 5.54; N, 21.64; S, 7.05

Viomycinic acid had a specific rotation of -24° (2.33 per cent water). The infrared spectrum of viomycinic acid had strong bands at 2.9, 6.0, and 9.0μ; medium bands at 3.2, 3.4, and 6.6μ; and weak bands at 4.8 and 7.4μ. All bands were broad. The nuclear magnetic resonance spectrum of a 24 per cent solution of viomycinic acid in deuterium oxide was recorded (Figure 3). The spectrum was traced on paper, and peaks were cut out and weighed. Assuming that the weight of the peak centered at 2.03τ corresponded to one proton, the weight of the peak centered at 6.07τ corresponded to 10.2 protons, that of the two peaks centered at 6.93 and 7.27τ corresponded to 7.8 protons, that of the peak centered at 7.77τ corresponded to 0.8 protons, and that of the peak centered at 8.22τ corresponded to 7.9 protons.

The Sakaguchi reaction of viomycinic acid and viomycin were compared. The procedure used was a slight modification of that of Vincent and Lagreu (25). Two milliliters of a solution of 30.5 mg. of the sample in 100 ml. water, 0.3 ml. of 10 N sodium hydroxide and 0.2 ml. of 0.1 per cent α-naphthol in absolute ethanol were mixed in a tube

and allowed to stand 15 minutes in an ice-water bath. Then 0.5 ml. of a solution of 0.5 ml. of bromine in 100 ml. of 2.5 N sodium hydroxide was added, and the tube was shaken and allowed to stand for 30 seconds in the ice-water bath. Then 0.5 ml. of 40 per cent aqueous urea and 3.0 ml. of 20 per cent by volume glycerol in ethanol were added. Viomycin appeared to give a more intense pink color than did viomycinic acid. The colors were compared with a Bausch and Lomb student model spectrophotometer at 520 mμ. The tube containing viomycin had an optical density of 0.244 while that containing viomycinic acid had an optical density of 0.071. Both optical densities were measured versus a reagent blank.

Acid Hydrolysis of Viomycinic Acid

A solution of 50 mg. of viomycinic acid in 2 ml. of 6 N hydrochloric acid was heated for six hours under reflux on a steam bath. Paper chromatograms of the hydrolysate were developed with BAW solvent and sprayed with ninhydrin. These chromatograms had spots whose R_F values and colors were as follows: 0.48, purple; 0.37, grey-blue; 0.32, pink; 0.29, orange at room temperature; and 0.17, green.

A solution of 211 mg. of viomycinic acid in 10 ml. of 6 N sulfuric acid was heated under reflux on a steam bath in a two-necked flask for six hours. Nitrogen, which had been bubbled through 5 N sodium hydroxide, was admitted through one neck and aerated the solution. The reflux condenser was placed in the other neck of the flask. The nitrogen passed out the top of the reflux condenser and passed through anhydrous calcium chloride and Drierite drying tubes. The gas then passed through a tube containing Ascarite. Only 4.6 mg. of

carbon dioxide was absorbed. In a blank determination the Ascarite gained 8.1 mg.

The hydrolysate was neutralized with IR-45 in the hydroxyl form and made alkaline by the addition of saturated barium hydroxide. The solution (about 50 ml.) was distilled to about 10 ml. into 50 ml. of five per cent boric acid. The ammonia was titrated with hydrochloric acid using screened methyl red as indicator. The titration required 14.05 ml. of 0.0248 N (0.348 millimoles) hydrochloric acid.

A solution of 100 mg. of viomycinic acid in 2 ml. of 6 N hydrochloric acid was allowed to stand at room temperature. Spots were applied to paper for chromatography at zero time and after 1, 2, 3, 4 and 6 days. The paper chromatograms were developed with BAW and sprayed with ninhydrin. The chromatograms had spots whose R_F values, colors, and intensities of color *versus* time were as follows: 0.36, grey-blue, faint after one day and increasing until six days; 0.50, purple, very faint after two days and increasing until six days; and 0.00, purple, intense for each application.

A solution of 200 mg. of viomycinic acid in 1 ml. of 6 N hydrochloric acid was allowed to stand at room temperature for three days. The hydrolysate was applied on a line 3 cm. from the bottom of a 13 x 40 cm. strip of thick paper (Whatman #17) for chromatography. The paper strip was developed with BAW to within 7 cm. of its top. The chromatogram was then allowed to dry, a thin slice cut, and the slice sprayed with ninhydrin reagent. An intense grey-blue band whose R_F value was 0.36, a faint purple band whose R_F value was 0.48, and an intense band at the origin resulted. The origin of the remainder of the

strip was cut out and eluted with water. Evaporation of the water *in vacuo* gave 120 mg. of tan solid.

Papergrams (BAW) of this material had a ninhydrin-positive spot at the origin. A solution of about 50 mg. of this material in 1 ml. of 6 N hydrochloric acid was heated on a steam bath under reflux overnight. Papergrams (BAW) of the hydrolysate had ninhydrin-positive spots whose R_F values and colors were as follows: 0.52, purple; 0.40, grey-blue; 0.36, pink; 0.33, orange at room temperature; and 0.19, green.

DNP-Viomycinic Acid

The DNP derivative of viomycinic acid was prepared by the same procedure as was bis-DNP-viomycin and bis-DNP-desureaviomycin. The yield was 295 mg., starting with 203 mg. of viomycinic acid. The DNP derivative was hydrolyzed with concentrated (*ca.* 12 N) hydrochloric acid in a pressure bottle as described for bis-DNP-viomycin. The residue was washed with ethyl acetate and paper chromatograms prepared. The ethyl acetate solution contained one yellow compound whose R_F values were 0.87 and 0.00 with BAW and phosphate buffer, respectively, as developing solvents.

Hydrazinolysis of Viomycinic Acid

A solution of 100 mg. of viomycinic acid in about 1.5 ml. of anhydrous hydrazine was treated as described for viomycin and desureaviomycin. Paper chromatograms developed with BAW and sprayed with ninhydrin had a faint purple spot whose R_F value was 0.48. There were other very faint spots.

Hydrolysis of Viomycin in Boiling Tap Water

A solution of 200 mg. of viomycin sulfate in 4 ml. of tap water was boiled under reflux on a sand bath. Aliquots were taken at zero time and after 4 hours, 3 days, 5 days, 10 days, 15 days, 18 days, 25 days and 40 days for paper chromatography and Nessler's test. The aliquots taken after three days or more of hydrolysis gave positive tests with Nessler's reagent. Paper chromatograms developed with BAW and sprayed with ninhydrin reagent had a purple spot whose R_F value was 0.00 for each aliquot. Chromatograms of aliquots taken after three days of hydrolysis and later had purple spots whose R_F value was 0.49-0.51. The intensity of the color of these spots increased until the fifteenth day of hydrolysis. Chromatograms of aliquots taken after five days and later had several purple spots whose R_F values were 0.30 and lower.

A solution of 6.85 g. of viomycin sulfate in 30 ml. of tap water was boiled under reflux on a sand bath for four weeks. The hydrolysate was applied to a column of 600 ml. of IR-50 that had been buffered at pH 7.0 with ammonium acetate and eluted until the effluent gave a very weak test with Nessler's reagent. The IR-50 column was eluted with water until the effluent gave a negative test with ninhydrin reagent. The solution was concentrated to a small volume by evaporation *in vacuo*. One hundred milliliters of IR-45 in the hydroxyl form was added to the solution. The mixture was evaporated to dryness and 100 ml. of absolute ethanol was added. The ethanol was evaporated *in vacuo*. The addition and evaporation of ethanol was repeated until the material gave a negative test with Nessler's reagent. The resin was washed with water

and the solution was evaporated to dryness. The resulting tan glass was dried *in vacuo* in a desiccator over calcium chloride for three days to give 1.24 g. Paper chromatograms of this material were developed with BAW solvent. A ninhydrin reagent-sprayed chromatogram had a large, intensely purple spot whose R_F value was 0.45 and a small purple spot whose R_F value was 0.63.

The IR-50 column was then eluted with 1 N sulfuric acid until the effluent gave a negative test with ninhydrin reagent. IR-45 in the hydroxyl form was added until the pH of the effluent rose to 8. The solution was then applied to a column of 560 ml. of IR-400 in the hydroxyl form. The IR-400 column was eluted with water until the effluent gave a negative test with ninhydrin and Nessler's reagent. The solution was evaporated to dryness and 100 ml. of IR-45 in the hydroxyl form was added. One hundred milliliters of absolute ethanol was added and evaporated *in vacuo*. The addition and evaporation of ethanol was repeated until the material gave a negative test with Nessler's reagent. The IR-45 was washed with water and the solution was applied to a column of 40 ml. of IR-45 in the sulfate form. The column was eluted with water until the effluent gave a negative test with ninhydrin reagent. The water was evaporated by lyophilization to give 3.71 g. of tan solid. Paper chromatograms of this material developed with BAW and sprayed with ninhydrin had an intense grey-blue spot whose R_F value was 0.21, a green spot whose R_F value was 0.09, and other faintly colored spots.

The IR-400 column was then eluted with 0.6 N hydrochloric acid until the effluent gave a negative test with ninhydrin reagent. IR-45

in the hydroxyl form was added until the pH of the effluent rose to 5. Paper chromatograms of this solution developed with BAW and sprayed with ninhydrin reagent had a purple spot whose R_F value was 0.24 and a purple spot at the origin. The solution was evaporated to dryness, by lyophilization, to give 0.56 g. tan solid.

The material eluted from the IR-50 column by acid had a specific rotation of 0.0° (5.29 per cent in water). A solution of this material in water had no peak in the ultraviolet range. This material, like viomycin, gave a negative test with o-aminobenzaldehyde.

A sample of this material was submitted for analysis. The analyst reported that the material gave a residue of 46 per cent after ignition. No further work was done with this material.

A solution of one gram of viomycin sulfate in 10 ml. of tap water was boiled under reflux. Aliquots were taken at zero time, and after 6 hours, 1 day, 2 days, 3 days, and 4 days. Each of the aliquots was evaporated to dryness by lyophilization and submitted for microbiological assay. The samples had the following activities towards Bacillus subtilis: zero time, 100 per cent (assumed); 6 hours, 41 per cent; 1 day, 15 per cent; 2 days, 1 per cent; 3 and 4 days, less than one per cent (19).

A solution of 9.80 g. of viomycin sulfate in 20 ml. of tap water was boiled under reflux for four days. The hydrolysate was evaporated to dryness by lyophilization to give 9.54 g. of pale yellow solid. A paper chromatogram of this material developed with BAW and sprayed with ninhydrin had a faint purple spot whose R_F value was 0.50 and an intense purple streak from the origin to R_F value ca. 0.20.

IR-45 in the hydroxyl form was added to a solution of this material until the pH rose to 8. The solution was then applied to a column of 80 ml. of IR-50 in the hydrogen form. The IR-50 column was eluted with water until the effluent gave a negative test with ninhydrin reagent. This solution was evaporated to dryness by lyophilization to give 3.04 g. of tan powder. A paper chromatogram of this material developed with BAW and sprayed with ninhydrin had a purple spot whose R_F value was 0.50 and a purple streak from the origin.

The IR-50 column was then eluted with 1 N sulfuric acid until the effluent gave a negative test with ninhydrin reagent. The effluent was neutralized with IR-45 in the hydroxyl form in a batch operation, and applied to a column of 50 ml. of IR-45 in the sulfate form. The solution was evaporated to dryness by lyophilization to give 5.22 g. of light tan powder. A paper chromatogram of this material that was developed with BAW and sprayed with ninhydrin had an intense purple streak from the origin.

Anal. $C_{22}H_{39}N_{11}O_9 \cdot 3/2\ H_2SO_4$
(748.7)

Calc'd: C, 35.29; H, 5.65; N, 20.58; S, 6.42

Found: C, 35.61; H, 5.64; N, 20.48; S, 7.31

The sample was dried to constant weight at 80° in vacuo; the loss was 5.94 per cent in 36 hours. Van Slyke primary amino nitrogen values were 3.89 per cent after 2.5 minutes, 4.55 per cent after 15 minutes, 4.71 per cent after 60 minutes, and 5.25 per cent after four hours.

The infrared spectrum of this material had strong bands at

2.9, 6.0, and 9.0μ; medium bands at 3.2, 3.4, 6.5, and 8.2μ; and weak bands at 4.8, 7.2, and 7.4μ. All bands were broad. The nuclear magnetic resonance spectrum of a 20 per cent solution of this material in deuterium oxide had peaks at 5.27, 6.00, 6.95, 7.20, and 8.20τ.

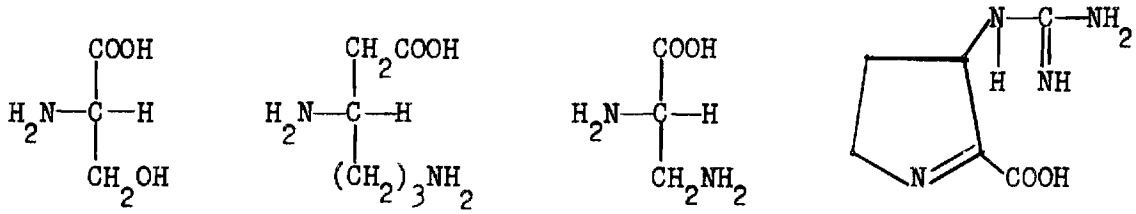
DISCUSSION

Review and Plans

Investigation of viomycin has shown that it is a polypeptide. The structure of a simple peptide or protein can be deduced in principle from the following information: the molecular weight of the simple peptide or protein; the identity and amount of each α-amino acid released on complete hydrolysis; the determination of C-terminal and N-terminal amino acid residues; and partial hydrolysis to peptides of lower molecular weight that can be identified.

The determination of the structure of a naturally occurring peptide often is complicated by structural features not found in simple peptides or proteins. Thus a peptide may have a cyclic structure, may be composed of amino residues other than the L-α-amino acid residues found in proteins, may have peptide bonds involving amino groups or carboxyl groups other than those at the α-position, and may have residues which are not amino acids.

Some of the information necessary for the determination of the structure of viomycin has been obtained. Its molecular weight has been determined. The acid hydrolysate of viomycin is known to contain L-serine (I), L-β-lysine (II), L-2,3-diaminopropionic acid (III), viomycidine (IV), urea, carbon dioxide, and ammonia. Of these compounds, only L-serine and ammonia are found in hydrolysates of proteins. The amounts of the amino acids present in the hydrolysate (Table 2), has been determined only by isolation. Viomycin is known to have no free carboxyl group.



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Viomycin has three basic centers; one of these is a guanidino group and the other two are primary amino groups. It has been shown that at least one of the primary amino groups of viomycin is one of the amino groups of the β-lysyl residue. One of the goals of the present research was to determine whether the β-amino group, the ε-amino group or both amino groups of the β-lysyl residue are free in the intact molecule. If only one of these amino groups is free, it would be necessary to determine whether it is the β-amino group or the ε-amino group. If this is the case, it also would be necessary to determine the position of the second primary amino group of viomycin.

Partial hydrolysis studies on viomycin have had only limited success. Viomycin was not affected by any of the common proteolytic enzymes with which it has been treated (26). Mild acid hydrolysis of viomycin has given promising results. The preparation of desurea-viomycin will be repeated and this material will be characterized further. Other conditions for partial hydrolysis of viomycin and desureaviomycin will be explored. The goal of partial hydrolysis studies is to obtain dipeptides and tripeptides whose identification will establish the order of peptide bonds in viomycin.

Knowledge of the order of peptide bonds in viomycin probably would be the most useful information for the determination of its

structure. However, any structure proposed for viomycin also will have to account for its other chemical properties and for its ultraviolet spectrum.

Viomycin

Preparation and Properties of Viomycin Sesquisulfate

Viomycin was received from Parke, Davis & Company as the neutral sulfate salt. This material was converted to the stoichiometric sulfate salt, viomycin sesquisulfate, by use of ion exchange resins.

Viomycin sesquisulfate was submitted for elemental analysis, van Slyke primary amino nitrogen determination, and determination of pK_a values. Analysis for carbon, hydrogen, nitrogen, and sulfur gave values that are more consistent with the formula $C_{25}H_{45}N_{13}O_{11} \cdot 3/2\ H_2SO_4$ than the accepted formula, $C_{25}H_{44}N_{12}O_{11} \cdot 3/2\ H_2SO_4$. The accepted formula will be used in all calculations. Van Slyke primary amino nitrogen values indicated that 1.22 primary amino groups react in 2.5 minutes, 1.98 groups react in 15 minutes, and 2.16 groups react in an hour. Haskell reported that 1.57 primary amino groups reacted in four minutes.

The ultraviolet spectra of solutions of viomycin sesquisulfate in water and in 0.1 N hydrochloric acid were recorded. The wave lengths and extinction coefficients of maximum absorption agree with reported values. The infrared spectrum of viomycin sesquisulfate had bands that indicated the presence of hydroxyl, amino, alkane, primary ammonium, amide carbonyl, and sulfate groups. The most interesting feature of nuclear magnetic resonance spectra of deuterium oxide solutions of viomycin sesquisulfate (Figure 1) is the absorption at 1.93τ. This

absorption corresponds to about 1/22 of the absorption by non-exchanging hydrogen atoms. Absorption in this range could be caused by formamide, formate, acetal, olefinic, aromatic, or aldehydic hydrogen atoms. Other absorptions correspond to those expected from the hydrolysis products of viomycin. Their assignments will be given later (Table 7).

The pK_a values obtained were 8.2 and 10.3; these are in agreement with values previously reported.

It had not been shown conclusively whether or not viomycin has an aldehydic or ketonic carbonyl group. Viomycin is reported to give a positive Fehling's test, but negative Benedict's and Tollen's test and no precipitate with either 2,4-dinitrophenylhydrazine or semicarbazide (1,2,4). When viomycin sesquisulfate was treated with Fehling's solution, a deep violet-colored solution resulted, but no precipitate formed. This test indicates that the report that viomycin gives a positive Fehling's test is in error. Viomycin gives a positive biuret test (deep violet color), and the reagents used in making biuret and Fehling's tests both involve alkaline copper sulfate.

One of the best methods for the detection of saturated aldehydes and ketones is based on their reaction with thiosemicarbazide. The formation of the thiosemicarbazone can be determined easily by ultraviolet spectroscopy. The thiosemicarbazone has a absorption peak at about 271 mμ of high intensity (ϵ, approximately 20,000) and a second peak at about 229 mμ (ϵ, approximately 7,500), while the carbonyl compound, in the absence of another chromophore, has only weak absorption in this region. Thiosemicarbazide itself has a peak at 241 mμ (ϵ, 13,800) (27). This method is especially useful in cases

of water-soluble compounds whose usual carbonyl derivatives, if formed, also might be water-soluble. The presence of the aldehydic carbonyl group of streptomycin was first demonstrated by Donovick, Rake, and Fried using this method (28). Their procedure was applied to viomycin. The ultraviolet spectrum showed that no thiosemicarbazone had formed. It is concluded that viomycin has no aldehydic or ketonic carbonyl group.

Δ^1-Pyrrolines and other imines give a yellow color when treated with o-aminobenzaldehyde in dilute acid. In the case of Δ^1-pyrrolines the color is stable for at least a day (18). Viomycidine gives stable yellow color with o-aminobenzaldehyde (11), while viomycin does not. This indicates that viomycin does not contain the Δ^1-pyrroline unit which has been suggested for viomycidine. This difference between viomycidine and viomycin is indicated also by their pK_a value. Thus viomycidine has a pK_a of 5.50 in water, while viomycin does not (11).

Acid Hydrolysis of Viomycin

A small amount of viomycin sulfate was hydrolyzed in concentrated acid for one-dimensional paper chromatography. It was hoped that a solvent that would give good resolution of spots would be found. Haskell reported that two-dimensional paper chromatograms developed using BAW in the first direction and phenol-water in the second, and sprayed with ninhydrin reagent, had five major spots and three minor ones. Four of the major spots corresponded to serine, β-lysine, viomycidine, and 2,3-diaminopropionic acid (6). Mason reported that similar chromatograms had six spots, one of which did not appear if the sample of hydrolysate applied was acidic. Four of these spots corresponded to serine, β-lysine, viomycidine, and 2,3-diaminopropionic acid (4).

Paper chromatograms of the hydrolysate were developed using several solvents. Of these solvents, BAW was found to give the best separation of spots and the least streaking or tailing. Chromatograms were spotted with serine, β-lysine, viomycidine, 2,3-diaminopropionic acid and the acid hydrolysate of viomycin, developed with BAW, and sprayed with ninhydrin and Weber reagents. Spots corresponding to compounds present in the hydrolysate were identified by comparison of their R_F values and colors to the R_F values and colors of the known compounds. A summary of the data is given in Table 3.

Table 3. Paper Chromatography of the Hydrolysate of Viomycin

Ninhydrin Reagent		Weber Reagent		Compound
R_F	Color	R_F	Color	
0.48	Purple	None		Serine
0.38	Grey-Blue	None		β-Lysine
0.32	Pink	0.32	Pink	Viomycidine
0.29	Orange (25°)	None		2,3-Diaminopropionic Acid
0.16	Green	0.16	Pink	Unknown

Identification of viomycidine and 2,3-diaminopropionic acid was difficult because their R_F values are similar. Their identification is possible, however, because only viomycidine gives a pink colored spot with Weber reagent and only 2,3-diaminopropionic acid gives a colored spot with ninhydrin reagent within a few minutes at room temperature. Since the R_F value of a given compound with a given solvent system can vary considerably with temperature and other factors, it is advisable to apply at least one known compound to each chromatogram before development. Serine usually was applied to chromatograms sprayed with

ninhydrin reagent, and viomycidine was applied to chromatograms sprayed with Weber reagent. It was found that when the R_F value of one compound was higher (or lower) than usual, the R_F values of other compounds were also higher (or lower) than usual.

A solution of viomycin sulfate in 6 N hydrochloric acid was allowed to stand at room temperature for two weeks. Paper chromatography indicated that urea, serine, and β-lysine were present in the hydrolysate after one day. The amount of serine increased until about the tenth day and the amount of β-lysine increased until about the fourth day. After about eleven days a complex pattern of ninhydrin-positive spots appeared on the chromatogram. No discrete spots corresponding to 2,3-diaminopropionic acid or to viomycidine were observed. These conditions of hydrolysis do not seem to cleave peptide bonds with much selectivity. However, the bonds which link urea, β-lysine, and serine in the molecule appear to be more easily cleaved under these conditions than those of viomycidine and 2,3-diaminopropionic acid. It is possible that viomycidine and 2,3-diaminopropionic acid are bound to each other.

Hydrogenation of Viomycin

Mason attempted to hydrogenate viomycin using two sets of conditions. The first attempt was made using Adams catalyst (29) under atmospheric pressure at 37°. The second was made using palladium on Norite under 42 lb./in.^{2} pressure at 25° for five hours. No alteration of the ultraviolet spectrum of viomycin resulted from the first attempt. A reduction of the extinction coefficient at maximum absorption by 20 per cent resulted from the second attempt (4).

An attempt to hydrogenate viomycin sesquisulfate in aqueous acetic acid using platinum on carbon as catalyst under 40 lb./in.^{2} at room temperature for three days was made. This attempt gave material whose microbiological activity was 70 per cent of that of viomycin sesquisulfate (19), whose extinction coefficient at maximum absorption (268 mμ) was 80 per cent of that of viomycin sesquisulfate, and whose nuclear magnetic resonance spectrum was very similar to that of viomycin sesquisulfate. These data indicate that viomycin has no group that is hydrogenated easily.

Ozonolysis of Viomycin

Mason showed that viomycin is oxidized by neutral permanganate with destruction of its ultraviolet chromophore. Hydrolysis of the oxidation product gave the same compounds as hydrolysis of viomycin itself. The destruction of the ultraviolet chromophore required four equivalents of permanganate. Mason concluded that this oxidation of viomycin involved the addition of hydroxyl groups to one or more olefinic double bonds (4).

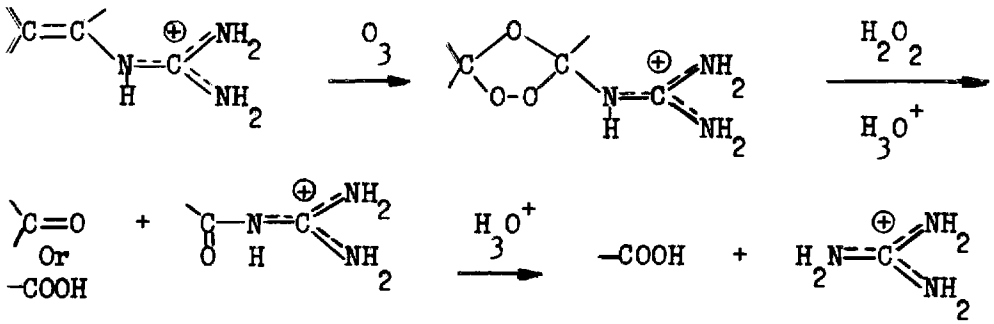
Ozone is a more specific reagent for olefinic double bonds than is permanganate. Unlike permanganate, ozone does not attack other easily oxidizable groups. When viomycin in cold aqueous acetic acid was treated with ozone and then with acidic hydrogen peroxide, it lost its ultraviolet chromophore. It was not determined whether the chromophore was destroyed by the preparation of the ozonide or by oxidative destruction of the ozonide with hydrogen peroxide.

Paper chromatograms of the solution after ozonolysis had only one ninhydrin-positive spot, which was at the origin. This indicated

that no cleavage of viomycin had taken place. Apparently the olefinic double bond(s) are in a cyclic residue. Otherwise oxidative destruction of the ozonide would have given two or more products.

The ozonolysis product was hydrolyzed in 6 N hydrochloric acid. Paper chromatograms indicated that serine, β-lysine, 2,3-diaminopropionic acid, viomycidine and at least two other guanidine compounds were present. The hydrolysate was separated into two fractions on a column of IR-400 in the hydroxyl form. Papergrams of the first fraction, which should contain strongly basic and non-ionic compounds, indicated that viomycidine, guanidine, and an unknown guanidine compound were present. Papergrams of the second fraction, which should contain acidic compounds and neutral and weakly basic amino acids, indicated that serine, β-lysine, 2,3-diaminopropionic acid and the unknown guanidine compound were present.

If guanidine is a major product of this series of reactions, then it is possible that viomycin has an ethylenic guanidine unit, as suggested by Mason. This unit would be expected to undergo the following reactions:



DNP-Viomycin

Sanger has shown that 2,4-dinitrofluorobenzene reacts with the amino groups of amino acids, peptides and proteins in aqueous ethanolic sodium bicarbonate solution at room temperature to give N-2,4-dinitrophenyl derivatives. In addition, 2,4-dinitrofluorobenzene reacts under these conditions with phenolic hydroxyl groups, sulfhydryl groups and with one of the imidazole nitrogens of histidine, but not with alkyl hydroxyl groups nor with guanidine nitrogens (20,21). The N-2,4-dinitrophenyl (DNP) derivatives of amino acids are bright yellow in color and usually can be obtained as crystalline, sharply melting solids.

Acid hydrolysis of a DNP-peptide or DNP-protein gives amino acids and the DNP derivative of the N-terminal amino acid residue(s) of the peptide or protein. This hydrolysis is always accompanied by some destruction of the DNP derivatives of the amino acid(s). The destruction of DNP derivatives of amino acids may be reduced by use of limited amounts of water. Thus 12 N hydrochloric acid gives less destruction of DNP-proline than does 6 N hydrochloric acid (22).

The DNP derivative of viomycin was prepared and hydrolyzed with 6 N hydrochloric acid as described by Mason (4). The acid hydrolysate was found to contain serine, viomycidine, 2,3-diaminopropionic acid, a second guanidine compound, and two yellow (DNP) compounds. One of these yellow compounds was shown to be 2,4-dinitrophenol. The other yellow compound was also ninhydrin-positive and may be a mono-DNP-β-lysine. In addition, the insoluble material produced in this hydrolysis was found to contain a compound whose R_F values on paper chromatograms were similar to those of bis-DNP-β-lysine.

It seemed likely that since 2,4-dinitrophenol was produced on 6 N hydrochloric acid hydrolysis of DNP-viomycin, from which any 2,4-dinitrophenol or 2,4-dinitrofluorobenzene had been completely removed by repeated washings with ether and ethanol, that some destruction of DNP derivatives occurred. This would account for the production of both bis-DNP-β-lysine and a mono-DNP-β-lysine. Presumably, bis-DNP-β-lysine is partially degraded to a mono-DNP-β-lysine and to 2,4-dinitrophenol.

Hydrolysis of DNP-viomycin with dioxane-concentrated hydrochloric acid and with concentrated (ca. 12 N) hydrochloric acid were studied using paper chromatography to determine if a hydrolysate free of 2,4-dinitrophenol and the mono-DNP-β-lysine could be obtained. The hydrolysis with concentrated hydrochloric acid seemed to give bis-DNP-β-lysine and no 2,4-dinitrophenol or mono-DNP-β-lysine. Results of paper chromatography of the concentrated hydrochloric acid hydrolysate are given in Table 4.

Table 4. Paper Chromatography of the Acid Hydrolysate of DNP-Viomycin

R_F Value	No Spray Reagent	Ninhydrin Color	Weber Color	Compound
0.17	None	Green	Pink	Unknown
0.29	None	Orange (25°)	None	2,3-Diaminopropionic acid
0.32	None	Pink	Pink	Viomycidine
0.46	None	Purple	None	Serine
0.84	Yellow	Yellow	None	Bis-DNP-β-lysine and/or 2,4-Dinitrophenol

This hydrolysis was repeated on a larger scale, the bis-DNP-β-lysine was purified by chromatography on silicic acid and its identity proven by its melting point and the melting point of a mixture of it and authentic bis-DNP-β-lysine. This proves that the two free primary amino groups of viomycin are those of the β-lysyl residue.

Sanger reported that the ultraviolet spectra of DNP derivatives of amino acids and peptides in dilute alkali solutions have peaks at 360 and 350 mμ, respectively. He suggested that concentrations of such solutions could be estimated by measurement of the absorption at 350 mμ using a value of 15,500 for the extinction coefficient (30). Battersby and Craig suggested that this method of estimation could be extended to estimate the molecular weight of the DNP derivative of a peptide or protein (31,32). Mason obtained the ultraviolet spectrum of DNP-viomycin in one per cent N,N-dimethylacetamide. He estimated the molecular weight of DNP-viomycin making the following assumptions: viomycin reacts to give bis-DNP-viomycin; each DNP group contributes 15,500 toward the extinction coefficient at 350 mμ; and bis-DNP-viomycin precipitates from the reaction mixture as the free base. He estimated that the molecular weight of bis-DNP-viomycin is 1312 and that of the free base of viomycin is 978 (4).

Other works have reported that some DNP-amino acids have ultraviolet spectra whose maxima are not at 360 mμ, and that extinction coefficients at maximum absorption of DNP-amino acids vary considerably. Also, the wavelength of and extinction coefficient at maximum absorption of a given DNP-amino acid or DNP-peptide vary from solvent to solvent (33,34). Probably the best estimate of the molecular weight of

a DNP-peptide is made by assuming that the extinction coefficient at maximum absorption of the DNP-peptide in a given solvent is equal to the extinction coefficient at that wavelength of the DNP derivative of the N-terminal amino acid of the peptide in the same solvent.

With the aim of obtaining a better estimate of the molecular weight of bis-DNP-viomycin, the following work was done. DNP derivatives of some amino acids were prepared and the ultraviolet spectra of their solutions in one per cent N,N-dimethylformamide and other solvents were obtained. The ultraviolet spectrum of DNP-viomycin in one per cent N,N-dimethylformamide was obtained. Table 5 gives the extinction coefficients at maximum absorption in the region 339 to 361 mμ of DNP-viomycin and the DNP derivatives of amino acids that were prepared. Estimates of the molecular weights of bis-DNP-viomycin and viomycin were made. It was assumed that the extinction coefficient at maximum absorption (259 mμ) of bis-DNP-viomycin was equal to the extinction coefficient (24,100) of bis-DNP-β-lysine at this wavelength. It also was assumed that bis-DNP-viomycin precipitates from the dinitrophenylation reaction mixture as the bicarbonate salt, which is converted to the monohydrochloride salt during washings with hydrochloric acid. This method gave the values 1150 for the molecular weight of bis-DNP-viomycin monohydrochloride and 782 for the molecular weight of the free base of viomycin. It must be remembered that this method of estimation does not give precise values of molecular weight. However, it does constitute a method that is entirely independent from other methods, such as the diaphragm-cell diffusion technique, which gave a value of 625 for the molecular weight of the free base of viomycin (4).

Table 5. Extinction Coefficients at Maximum Absorption of DNP Derivatives[a]

Compound	In Glacial Acetic Acid[b]	In One Per Cent DMF[c,d]	In 1 N Sodium Hydroxide[e]
DNP-L-Serine	16,300 (16,100)[f]	15,500	16,800[g] (17,300)[g,h]
DNP-β-Alanine		18,900	17,200 (15,800)[h]
DNP-DL-Aspartic Acid	16,600 (16,100)[f]	17,700	17,900
Bis-DNP-β-Lysine		26,400	
Bis-DNP-Viomycin		26,100[i]	
Bis-DNP-Desureaviomycin		26,100[i]	

a. Values in parentheses are from the literature.
b. The maxima are at 339-342 mμ.
c. The abbreviation DMF is for N,N-dimethylformamide.
d. The maxima are at 356-361 mμ.
e. The maxima are at 357-361 mμ.
f. Value is from Schroeder and Legette (35).
g. In this case the solvent is four per cent sodium bicarbonate.
h. Value is from Rao and Sober (22).
i. Value is assumed.

A value of 782 for the molecular weight of the free base of viomycin corresponds to 929 as the molecular weight of the sesquisulfate.

Desureaviomycin

Preparation and Properties

Desureaviomycin hydrochloride was prepared as by Mason (4) and further investigation of it was made. Neither desureaviomycin hydrochloride nor desureaviomycin sulfate could be crystallized from aqueous ethanol or aqueous acetone. The stoichiometric sulfate salt of desureaviomycin was submitted for elemental analysis and van Slyke primary amino nitrogen determination. Analysis for carbon, hydrogen, nitrogen, and sulfur gave values that were consistent with the formula

$C_{24}H_{40}N_{10}O_{10} \cdot 3/2\ H_2SO_4$. This formula represents subtraction of the elements of urea from the accepted formula of viomycin sesquisulfate. Van Slyke amino nitrogen values indicated that 1.94 primary amino groups reacted in 2.5 minutes, 2.22 groups reacted in 15 minutes, and 2.56 groups reacted in an hour. These values indicate that no primary amino groups are released in the conversion of viomycin to desurea-viomycin.

Mason reported that when a solution of viomycin in 0.1 N hydrochloric acid was heated at 95° the maximum in absorption was at 267 mμ (ε, 23,200) at zero time, at 268 mμ (ε, 7,450) after six hours, at 268 mμ (ε, 4,880) after seven hours, at 272 mμ (ε, 9,200) after 12 hours, and at 274 mμ (ε, 7,200) after 24 hours (4). When this procedure was repeated, the maximum in absorption was at 267 mμ (ε, 27,200) at zero time and at 268 mμ (ε, 5,500) after six hours. The maximum remained at 268 mμ and its extinction coefficient continued to decrease slowly up to 24 hours (26). Since urea is cleaved quantitatively from viomycin in six hours, under these conditions, and since the decrease in the extinction coefficient continues after six hours, urea probably is not involved in the ultraviolet chromophore of viomycin.

Desureaviomycin hydrochloride had a specific rotation of -31.8° (five per cent in water). The infrared spectrum of desureaviomycin sulfate was very similar to that of viomycin sulfate. The nuclear magnetic resonance spectrum of desureaviomycin sulfate (Figure 2) also was very similar to that of viomycin sulfate. The peak at 1.92 τ corresponds to 1/22 of the absorption by non-exchanging protons. Other absorptions correspond to those expected from the hydrolysis products

of viomycin. Their assignment will be given later (Table 7).

DNP-Desureaviomycin

The DNP derivative of desureaviomycin was prepared by the same procedure as was DNP-viomycin. DNP-Desureaviomycin blackened when heated above 200°. Hydrolysis of DNP-desureaviomycin with concentrated (ca. 12 N) hydrochloric acid gave serine, 2,3-diaminopropionic acid, viomycidine and another guanidine compound in solution. Yellow, acid-insoluble material precipitated during the hydrolysis. This material was purified by chromatography on a silicic acid column and was shown to be bis-DNP-β-lysine by its melting point and the melting point of a mixture of it and authentic bis-DNP-β-lysine. This shows that no primary amino groups are released in the hydrolysis of viomycin to desureaviomycin. The ultraviolet spectrum of DNP-desureaviomycin in one per cent N,N-dimethylformamide was obtained. The molecular weight of bis-DNP-desureaviomycin monohydrochloride was estimated to be 1120 by the method used to estimate the molecular weight of bis-DNP-viomycin monohydrochloride. The molecular weight of the free base of desureaviomycin was estimated to be 752 and that of the sulfate salt was 896.

Hydrazinolysis of Desureaviomycin

It is possible that the hydrolysis of viomycin to desureaviomycin and urea involves the cleavage of an acyl urea. If this were the case, desureaviomycin should have a free carboxyl group. Two methods were used to determine whether or not desureaviomycin has a free carboxyl group. These methods were treatment with anhydrous hydrazine and carboxypeptidase.

Boiling anhydrous hydrazine reacts with a peptide or protein to

give amino acid hydrazide(s) and the C-terminal amino acid(s) of the peptide or protein. The reaction involves hydrazinolysis of peptide bonds. The excess hydrazine is removed and the amino acid(s) and the amino acid hydrazide(s) are identified by paper chromatography. Identification of the amino acid(s) is simplified by removal of the amino acid hydrazide(s). These are removed by dissolving the hydrazinolysate, free of hydrazine, in water, adjusting the pH of the solution to 7, and adding benzaldehyde. This treatment converts amino acid hydrazides to their benzylidene derivatives which are insoluble in water. This leaves only the amino acid(s) in solution (36). This method was applied to desureaviomycin sulfate. Paper chromatography of the hydrazinolysate, after the removal of amino acid hydrazides, showed that serine and no other amino acid was present. This indicates that the carboxyl group of a seryl residue in desureaviomycin is free. When this method was applied to viomycin, no serine or other amino acid was present in the hydrazinolysate.

Viomycinic Acid

The enzyme carboxypeptidase cleaves the C-terminal α-amino acid(s) from a peptide or protein. If this cleavage leaves the peptide or protein with a new C-terminal α-amino acid, then it too can be cleaved. Thus the enzyme is capable of splitting a peptide or protein to α-amino acids one residue at a time. The action of carboxypeptidase is rather general, but certain structural features of peptides or proteins impede its action. For example, it will not cleave a C-terminal α-amino acid residue when the residue is proline or is directly attached to a prolyl residue. Also, the C-terminal residue and the residue to which it is

attached both must be L-α-amino acids. A suspension of pancreatic carboxypeptidase was added to a solution of desureaviomycin buffered at pH 7.5 and the mixture was allowed to stand at room temperature for 20 days. Paper chromatograms of the solution indicated that serine was released during the first two days. Only traces of other ninhydrin-positive compounds appear on the chromatograms after 20 days. This indicates that the carboxyl group of a seryl residue of desureaviomycin is free. The fact that no other amino acid is released implies that the seryl residue is bound to a residue that is not attacked by carboxypeptidase.

Treatment of desureaviomycin with carboxypeptidase was repeated on a larger scale in order to allow separation and isolation of the products. Serine was separated from the other product of the cleavage, called viomycinic acid, on a column of IR-50 in the hydrogen form. The separation was incomplete since the water eluate of the column contained serine and some viomycinic acid. The acid eluate of the column contained only viomycinic acid and this material was used in the investigation of viomycinic acid.

A sample of viomycinic acid was submitted for elemental analysis and van Slyke primary amino nitrogen determination. Analysis for carbon, hydrogen, nitrogen, and sulfur gave values that were only fairly consistent with the formula $C_{21}H_{35}N_9O_8 \cdot 3/2\ H_2SO_4$. This formula represents replacement of a seryl residue of desureaviomycin by a hydroxyl group. Van Slyke amino nitrogen values indicated that 2.11 primary amino groups react in 2.5 minutes, 2.31 groups react in 15 minutes, and 2.39 groups react in an hour. The van Slyke primary amino nitrogen values of viomycin,

desureaviomycin, viomycinic acid and some known compounds *versus* time are given in Table 6.

Table 6. Van Slyke Amino Nitrogen Values[a]

Compound	2.5 Minutes	15 Minutes	60 Minutes
Viomycin	1.22	1.98	2.16
Desureaviomycin	1.94	2.22	2.56
Viomycinic Acid	2.11	2.31	2.39
Acetylurea	0.060	0.068	0.069
Acetylguanidine	0.29	0.34	0.37
2-Aminoimidazoline		0.26[b]	1.02[b]
2-Aminoimidazoline-4,5-dicarboxylic acid		0.88[b]	1.87[b]
Arginine		1.01[c]	1.06[c]

a. Values are moles of nitrogen per mole of compound.
b. Value is from the literature (37).
c. Value is from the literature (38).

The specific rotation of viomycinic acid was -24° (2.33 per cent in water). The infrared spectrum of viomycinic acid was very similar to those of viomycin sulfate and desureaviomycin sulfate. The nuclear magnetic resonance spectrum of viomycinic acid (Figure 3) also was similar to those of viomycin sulfate and desureaviomycin sulfate. The peak at 2.03τ corresponds to 1/28 of the absorption of the non-exchanging protons. Correlation of the τ values of viomycin, desurea-viomycin, viomycinic acid, serine, β-lysine, 2,3-diaminopropionic acid, and viomycidine is given in Table 7.

The intensity of the Sakaguchi reaction of viomycinic acid was found to be about 30 per cent of that of viomycin sulfate.

Table 7. τ-Values of Viomycin and Hydrolysis Products

Compound	Number of Protons				
	1.9 - 2.1 τ	4.0 - 5.6 τ	5.7 - 6.4 τ	6.5 - 7.6 τ	8.0 - 8.4 τ
Viomycin	1[a]	[b]	7.4	6.8	6.5
Desureaviomycin	1[a]	[b]	8.1	6.2	6.5
Viomycinic Acid	1[a]	[b]	10.2	7.8	7.9
L-Serine[c]	0	1	2	0	0
L-β-Lysine[c]	0	0	0	5	4
L-2,3-Diaminopropionic Acid[c]	0	1	2	0	0
Viomycidine[c]	0	3	0	2	0

a. Value is assumed.

b. Absorption in this region, which includes exchangeable protons, was off scale.

c. τ-Values are assigned on the basis of those of the amino acid and available peptides.

Hydrolysis of viomycinic acid with boiling 6 N hydrochloric acid gave serine, β-lysine, 2,3-diaminopropionic acid, viomycidine, and another guanidine compound. Hydrolysis of viomycinic acid with 6 N sulfuric acid gave no carbon dioxide. The fact that this material released no carbon dioxide was surprising, since desureaviomycin gives a mole of carbon dioxide under similar conditions. The sulfuric acid hydrolysate contained an amount of volatile base, presumably ammonia, equal to one mole per mole of viomycinic acid.

A solution of viomycinic acid in 6 N hydrochloric acid was allowed to stand at room temperature for several days. Paper chromatograms of this solution indicated that β-lysine was released during the first three days and that serine was released from the third to the sixth days. A solution of viomycinic acid in 6 N hydrochloric acid was allowed to stand at room temperature for three days. The products of the hydrolysis were separated by chromatography on thick

paper. The chromatogram was cut and the material which remained at the origin was eluted with water. Hydrolysis of this material with 6 N hydrochloric acid at 95° gave serine, β-lysine, 2,3-diaminopropionic acid and viomycidine. The fact that β-lysine was produced in this hydrolysis shows that the release of β-lysine from viomycinic acid by treatment with 6 N hydrochloric acid at room temperature was incomplete.

The DNP derivative of viomycinic acid was prepared and hydrolyzed with concentrated hydrochloric acid as described for bis-DNP-viomycin and bis-DNP-desureaviomycin. The acid solution contained serine, 2,3-diaminopropionic acid, viomycidine and another guanidine compound. Paper chromatography of the yellow, acid-insoluble material showed that it contained bis-DNP-β-lysine as the only ethyl acetate-soluble DNP compound. This indicates that no primary amine groups are released in the conversion of desureaviomycin to viomycinic acid.

The fact that serine is present in the hydrolysates of viomycinic acid and its DNP derivative indicates that viomycin and desureaviomycin contain at least two seryl residues. The isolation of 1.3 moles of serine per mole of viomycin from hydrolysates of viomycin also indicates that viomycin has two or more seryl residues (4,5).

Viomycinic acid was treated with anhydrous hydrazine as described for desureaviomycin. Paper chromatograms of the hydrazinolysate, after removal of hydrazine and amino acid hydrazides, showed that serine and no other amino acid was present. This result indicates that serine is the C-terminal amino acid residue of viomycinic acid as well as of desureaviomycin.

Degradation of Viomycin in Boiling Tap Water

When a solution of viomycin at pH 5-6 was boiled, its half-life, as measured by microbiological assay, was 12 hours (1). When a solution of viomycin sulfate in tap water (pH ca. 4.5) was boiled, the microbiological activity was reduced to 41 per cent after six hours, to 15 per cent after one day, to one per cent after two days, and to less than one per cent after three days (19). Attempts to determine what changes in the molecule accompany this loss of microbiological activity were made. It was found that the intensity of the Sakaguchi reaction decreased to a minimum value in two days, that the ultraviolet chromophore was destroyed in four days, and that a mole of ammonia was released in eight days (26). Paper chromatography showed that serine is present after three days.

A solution of viomycin sulfate in tap water was boiled for four days. Serine was separated from the other product(s) of this treatment. This other product was submitted for analysis of the elements and van Slyke primary amino nitrogen determination. Analysis for carbon, hydrogen, nitrogen, and sulfur gave values that are consistent with the formula $C_{22}H_{39}N_{11}O_9 \cdot 3/2\ H_2SO_4$. This formula represents replacement of a seryl residue of viomycin by a hydroxyl group. Van Slyke amino nitrogen values were 2.06 moles after 2.5 minutes, 2.44 moles after 15 minutes, and 2.52 moles after an hour. The infrared spectrum of this material was similar to that of viomycin sulfate. The nuclear magnetic resonance spectrum of this material also was similar to that of viomycin sulfate, except that the peak at 1.93τ is absent.

CONCLUSIONS

The accepted formula of viomycin sulfate is $C_{25}H_{44}N_{12}O_{11}\cdot 3/2\ H_2SO_4$ (MW, 836). As noted by Mason, addition of the formulas of the known hydrolysis fragments gives only 20 carbon atoms. Isolation of an excess of one mole of serine from hydrolysates indicated that viomycin has two seryl residues. Addition of another formula of serine gives 23 carbon atoms, but 13 nitrogen atoms. This addition is given below.

Serine (two moles)	$C_6H_{14}N_2O_6$
β-Lysine	$C_6H_{14}N_2O_2$
2,3-Diaminopropionic Acid	$C_3H_8N_2O_2$
Viomycidine	$C_6H_{10}N_4O_2$
Urea	CH_4N_2O
Carbon Dioxide	CO_2
Ammonia	H_3N
	$C_{23}H_{53}N_{13}O_{15}$

Subtraction of six moles of water, one for each carboxyl group and one for carbon dioxide, gives $C_{23}H_{41}N_{13}O_9$ (alternate formula I). Mason suggested that a single nitrogen of viomycin might be distributed between two of its hydrolysis products. The possibility that the same residue gives urea and ammonia is eliminated by the fact that desurea-viomycin gives a mole of ammonia on hydrolysis. However, it is possible that two amino acids share a common nitrogen atom. Isolation or other determination of greater amounts of each of the amino acids of the hydrolysate than have been reported (Table 2) would exclude this

possibility. These determinations would be laborious at best, but still might be worth the effort. They also would establish whether viomycin has two or more residues of serine. If two of the amino acids do share a common nitrogen atom, the formula of viomycin might be $C_{23}H_{38}N_{12}O_9 \cdot 3/2\ H_2SO_4$ (alternate formula II).

It would seem that a choice from the accepted formula of viomycin, alternate formula I, and alternate formula II could be made on the basis of analytical data and the molecular weight. Table 8 gives values of carbon, hydrogen, nitrogen, and sulfur that have been reported, and values calculated from these formulas. The ratio of carbon to nitrogen also is given in Table 8 because this number is independent of the dryness of the sample and the stoichiometry of the sulfate salt.

Table 8. Per Cent Composition of Viomycin Sesquisulfate

	C	H	N	S	C/N
Accepted Formula	35.93	5.67	20.11	5.75	1.79
Alternate I	34.92	5.61	23.03	6.08	1.52
Alternate II	35.70	5.34	21.72	6.22	1.70
Reported (1)	37.19	5.86	20.61	5.88[a]	1.80
Reported (2)	35.83	5.77	21.08	5.34	1.70
Reported (6)	35.89[b]	5.52[b]	21.15[b]	5.79[b]	1.70[b]
Reported (4)	34.22[b]	5.73[b]	21.85[b]	4.73[b]	1.56[b]
This Work	35.01	6.19	21.01	5.73	1.67
Average of Found	35.71	5.83	21.19	5.51	1.69

a. Determined as sulfate.
b. Not corrected for residue after ignition (1.25 per cent).

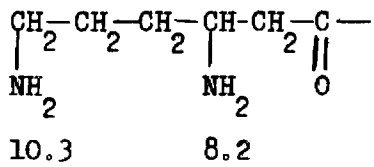
The reported analytical data probably eliminate alternate formula I, but they do not strongly favor either the accepted formula or alternate formula II.

If the accepted formula of viomycin is correct, the hydrolysate, including two moles of serine, does not account for two carbon atoms. Viomycin has no C-methyl group and its hydrolysate contains no volatile acids or carbonyl compounds. Thus the possibility that the hydrolysate of viomycin contains a C_2 compound or two C_1 compounds that have escaped detection probably is eliminated. This discrepancy of two carbon atoms is the objection to the accepted formula that led to the proposals of alternate formulas I and II.

Mason concluded that the value (772) he obtained by the diaphragm-cell diffusion technique does not refute the accepted formula of viomycin sulfate (MW, 836) (4). The molecular weights of the sulfate salts of alternate formulas I and II are 791 and 774, respectively. These values are much closer to the observed value than is that of the accepted formula. The value (930) of the molecular weight of viomycin sulfate obtained from the absorption at 357 mμ by bis-DNP-viomycin is too unreliable to eliminate or substantiate any of the formulas discussed.

Neither the accepted formula nor the alternate formulas agree very well with both the analytical data and the diffusion molecular weight value. The best course seems to be to use the accepted formula with reservations until it or some other formula is better established.

Preparation and hydrolysis of bis-DNP-viomycin showed that both amino groups of the β-lysyl residue of viomycin are free. Values of pK_a are assigned to these amino groups by comparison to known pK_a values of amino acids and peptides as shown below.

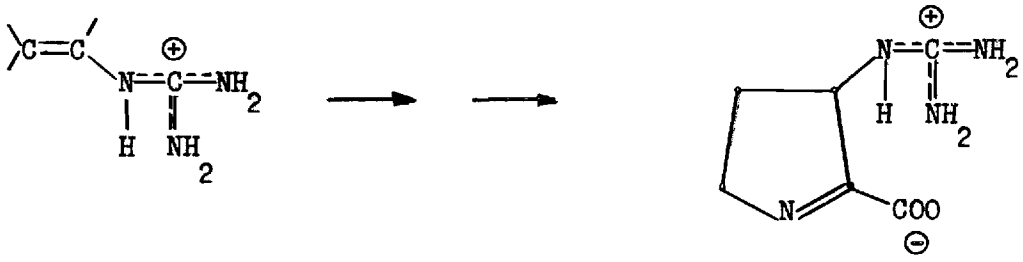


Van Slyke amino nitrogen values of 1.22 moles after 2.5 minutes, 1.57 moles after 5 minutes (6), and 1.98 moles after 15 minutes also are consistent with this structure. Amino groups other than those at the α-position give a mole of nitrogen more slowly (7-15 minutes) than those at the α-position (4-5 minutes) (39). The van Slyke amino nitrogen value of 2.16 moles of nitrogen after an hour indicates that either slow hydrolysis of peptide bonds occurs or that some other group reacts slowly to give nitrogen. Hydrazinolysis and pK_a values show that viomycin has no free carboxyl group. Negative results with thiosemicarbazide and other diagnostic reagents show that viomycin has no aldehydic or ketonic carbonyl group. Negative results with o-aminobenzaldehyde reagent and absence of a weakly basic center indicate that viomycin does not have a Δ^1-pyrroline structure. Positive Sakaguchi tests and the pK_a value of 12 indicate that viomycin has a guanidine group. Existing information does not suggest a structure for the residue of viomycin that is the precursor of viomycidine.

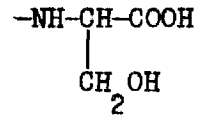
Preliminary results of oxidation of viomycin by permanganate (4) and ozonolysis indicate that an olefinic double bond may be present. Destruction of the ultraviolet chromophore of viomycin by oxidation and ozonolysis further indicates that this double bond, if present, is involved in the ultraviolet chromophore. Further study of ozonolyses of viomycin is indicated. It should be determined whether or not the ultraviolet chromophore is destroyed by ozone only. Also reductive,

as well as oxidative, destruction of the ozonide could be explored. Finally, the identity and amounts of compounds, especially guanidine and viomycidine, in hydrolysates of the ozonolysis products should be determined. Treatment of viomycin under conditions for hydrogenation reduced its microbiological activity and extinction coefficient at maximum absorption by 20-30 per cent. It might be worthwhile to treat viomycin under these conditions for extended periods of time to see whether or not it loses its microbiological activity and/or ultraviolet chromophore. Study of the possible hydrogenation product and its hydrolysate might give valuable information.

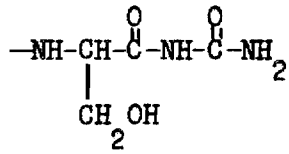
Alteration of the ultraviolet chromophore with pH, and parallel changes in intensities of Sakaguchi reaction and destruction of the ultraviolet chromophore suggest that the guanidine group is involved in the chromophore. Mason suggested that viomycin may have an ethylenic guanidine structural unit (4). The carbon atom that bears the guanidine group in the proposed structure for viomycidine (IV) is asymmetric. If viomycin contains an α,β-unsaturated guanidine function, then the α-carbon atom is not asymmetric. Because there are many asymmetric centers in viomycin, some possibly close to the proposed α,β-unsaturated guanidine function, it is possible that an asymmetric atom α to the guanidine function can arise by hydrolysis or rearrangement.



The procedure of Mason for the preparation of desureaviomycin (4) was repeated. Hydrazinolysis of desureaviomycin showed that the carboxyl group of a seryl residue is free.

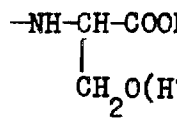


It is likely that urea was released from this seryl residue. If this is true, viomycin would have the partial structure

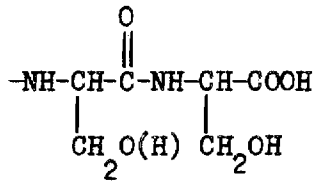


Van Slyke amino nitrogen values and the preparation and hydrolysis of the 2,4-dinitrophenyl derivative of desureaviomycin show that both amino groups of its β-lysyl residue are free and that no other primary amino groups are present. Therefore, no peptide bonds are cleaved in the conversion of viomycin to desureaviomycin.

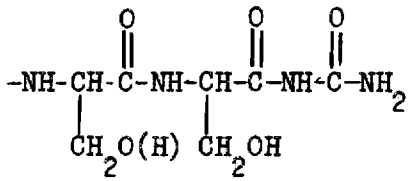
New conditions for partial hydrolysis of viomycin and desureaviomycin were explored. The most promising of these was treatment of desureaviomycin with carboxypeptidase. Serine was released to give a new material, called viomycinic acid. Hydrazinolysis of viomycinic acid indicated that the carboxyl group of a seryl residue was free. If this is true, viomycinic acid would have the partial structure



desureaviomycin would have the partial structure



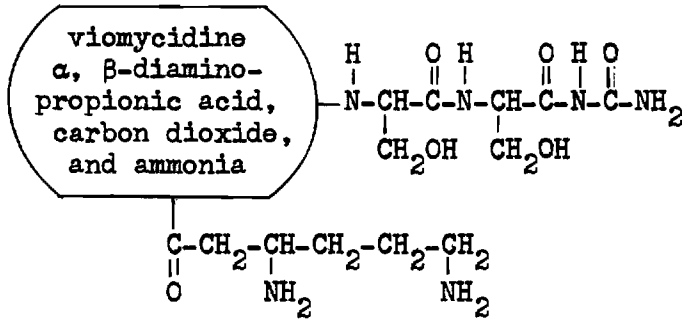
and viomycin would have the partial structure



Preparation and hydrolysis of the DNP-derivative of viomycinic acid showed that both amino groups of β-lysyl residue are free. Van Slyke primary amino nitrogen values for viomycinic acid are higher than those of viomycin and desureaviomycin. Acid hydrolysis of viomycinic acid gave serine, β-lysine, viomycidine, 2,3-diaminopropionic acid and ammonia, but little or no carbon dioxide. Treatment of viomycinic acid with hydrochloric acid at room temperature released β-lysine more rapidly than serine and did not release viomycidine or 2,3-diamino-propionic acid. Under these conditions, acyl groups migrate from the amino group of seryl and threonyl residues to the hydroxyl group. Hydrolysis of the resulting esters is more rapid than hydrolysis of amides. In the case of viomycinic acid, these reactions would give serine. The observation that β-lysine was released, by direct hydrolysis of a peptide bond, more rapidly than serine was released indicates that the N-acyl to O-acyl migration did not occur in viomycinic acid. It is possible that some structural feature, e.g.

substitution on the hydroxyl oxygen of the seryl residue, prevents the migration.

Those portions of the viomycin molecule that have been established as a result of this work may be summarized in the following partial structural formula. The nature of the linkages



giving rise to the hydrolysis products contained within the circle remain at present unknown.

Since it has not been established that the substances desurea-viomycin and viomycinic acid are single, pure compounds, results of qualitative procedures are somewhat uncertain. These materials should be analyzed by powerful methods of separation, e.g. counter-current distribution and chromatography.

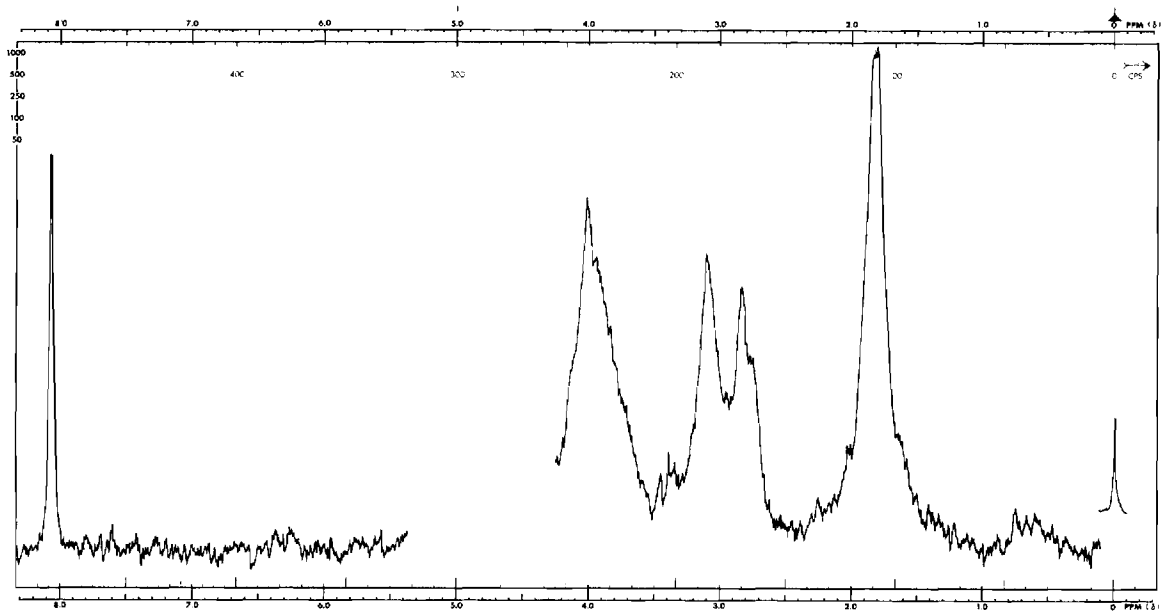


Figure 1. Nuclear Magnetic Resonance Spectrum of Viomycin Sulfate in Deuterium Oxide Solution.

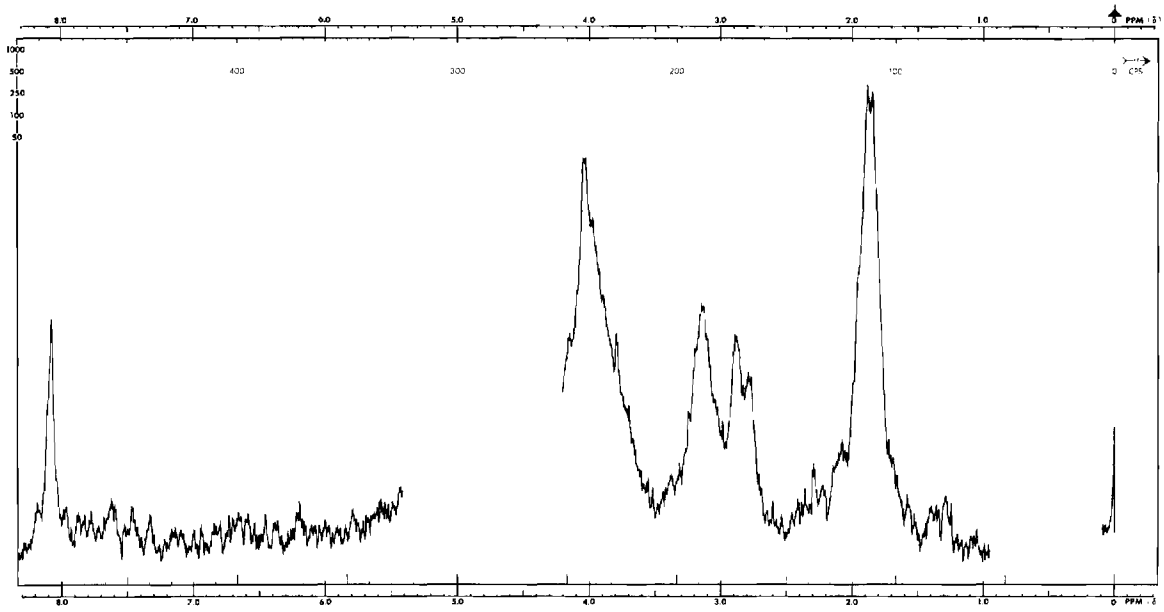


Figure 2. Nuclear Magnetic Resonance Spectrum of Desureaviomycin Sulfate in Deuterium Oxide Solution.

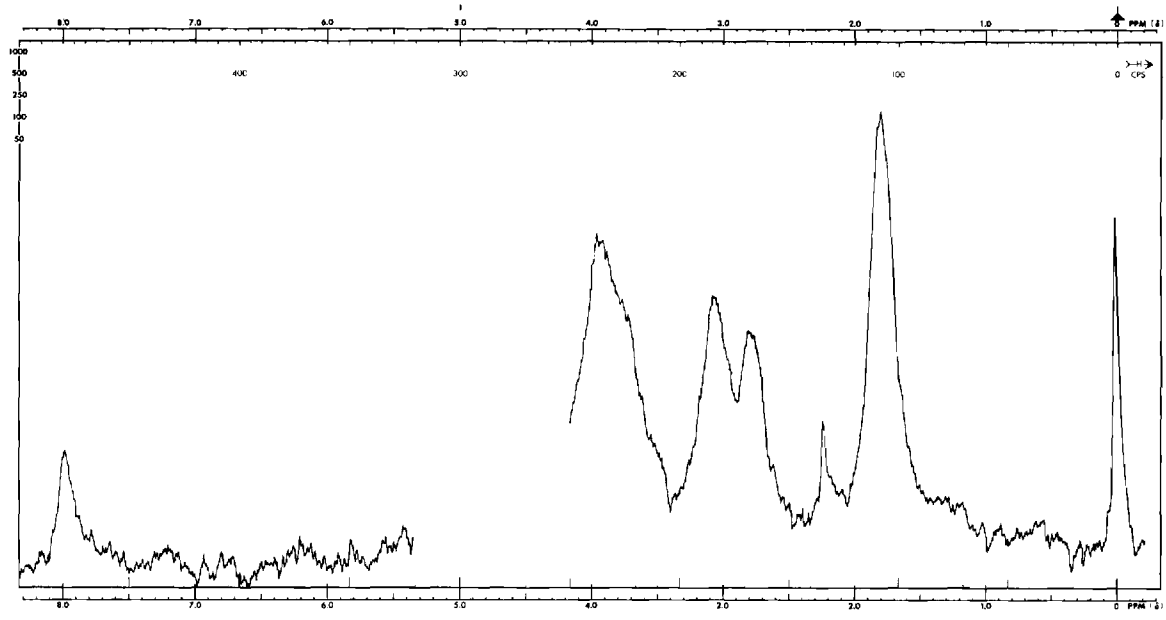


Figure 3. Nuclear Magnetic Resonance Spectrum of Viomycinic Acid in Deuterium Oxide Solution.

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*Abbreviations used are those suggested by Chemical Abstracts.

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VITA

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